Mothers and Forgotten Fathers

Prenatal effects and paternal influences on mammalian sex ratios

by

Tiana Pirtle, BSc (Hons)

School of Natural Sciences – Biological Sciences

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Declarations by the author

Declaration of Originality

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The research associated with this thesis abides by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition, 2004 and the University of Tasmanian Animals Ethics Guidelines. This research was conducted under University of Tasmania Animal Ethics Approval A0014877, A0012366, and A0015988.

I acknowledge and agree to the above stated declarations.

Signed:

Date: 5/8/2020

Statement of Contribution

The candidate was the lead researcher and author of all work presented in this thesis. The following people and institutions who contributed to the preparation of each data chapter are listed below.

Chapter 2 was conceived by T. Pirtle, E. Cameron, E. Wapstra, L. Parsley, J. McEvoy and A. Edwards. A. Edwards, and T. Gibbons were involved in animal care and behavioural testing. T. Pirtle analysed the data and led the writing of the manuscript, with contributions from E. Cameron, E. Wapstra, and L. Parsley.

Chapter 3 was conceived by T. Pirtle, E. Cameron, E. Wapstra, and L. Parsley. Protocols were developed by T. Pirtle, L. Parsley, T. Pinfold, and A. Smolenski. Samples were acquired by T. Pirtle, L. Parsley, Z. Gibb, A. Clutton-Brock, A. Swanson, and L. Morris. T. Pirtle and L. Parsley analysed samples. Data were analysed by T. Pirtle. T. Pirtle led the writing of the manuscript, with contributions from E Cameron, E. Wapstra, and L. Parsley.

Chapter 4 was conceived by T. Pirtle, E. Cameron, E. Wapstra, and L. Parsley. D. Baker, J. Ransom, B. McCann, M. Weber and T. Pirtle collected the original data. T. Pirtle collated and analysed the data. T. Pirtle led the writing of the manuscript, with contributions from E. Cameron, E. Wapstra, and L. Parsley.

Chapter 5 was conceived by T. Pirtle, E. Cameron, E. Wapstra, and L. Parsley. Data was collected by E. Cameron, T. Pirtle, A. Edwards, and S. Rainer. T. Pirtle analysed the data and led the writing of the manuscript with contributions from E. Cameron, E. Wapstra, and L. Parsley.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the manuscripts contributing to this thesis:

Signed:

Date: 7 August 2020

Professor Elissa Cameron
Primary Supervisor
School of Natural Sciences
University of Tasmania

7 August 2020

Professor Greg Jordan
Head of Discipline
School of Natural Sciences
University of Tasmania

Signed:

Date: 6 August 2020

Associate Professor Erik Wapstra
Supervisor
School of Natural Sciences
University of Tasmania

7 August 2020

Dr Laura Parsley
Supervisor
School of Natural Sciences
University of Tasmania

Tiana Pirtle
Candidate
School of Natural Sciences
University of Tasmania

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Notes on Text

This thesis is compiled of a series of chapters prepared as standalone papers for publications.

Thus, there is some repetition, particularly among the introductions of each data chapter.

Abstract

Sex allocation theory predicts that parental fitness is enhanced when parents adjust offspring sex ratios according to the relative fitness returns from sons and daughters. Compared to other taxa, research in mammals exhibits inconsistent empirical results relative to theory. Such inconsistencies could, in part, relate to the degree of prenatal masculinisation or feminisation of female phenotypes altering capacity to adjust sex ratios in adulthood. Further, fathers might also influence offspring sex ratios through sperm sex ratios and seminal plasma composition. My thesis addressed the effects of prenatal masculinisation and feminisation on maternal sex ratios and the scope of paternal influences on sex ratios. I first tested how the degree of prenatal masculinisation or feminisation of female mice (*Mus musculus*) influenced offspring sex ratios. I found that more feminised females produced more daughters. Thus, I suggest that some inconsistencies between theory and observation in mammalian sex allocation relate to phenotypic variation among females relating to prenatal experience. Secondly, I tested the influence of fathers on sex ratios by quantifying sperm sex ratios in frequently and infrequently mating domestic stallions (*Equus ferus caballus*). I increased the mating frequency of all stallions to induce sexual exhaustion over three days, mimicking feral stallion mating frequencies. Sperm sex ratios were not at parity. The proportions of X-chromosome-bearing-spermatozoa (CBS) increased in successive ejaculates in frequently mating stallions, while the infrequently mating stallions maintained an X-CBS bias throughout the experimental period. I suggested that Y-CBS are more rapidly depleted than X-CBS with sexual exhaustion in frequently mating stallions. Third, I measured key components of seminal plasma: sex hormones, glucose, major cations and trace elements, and their relationship to X- and Y-CBS in successive ejaculates. Seminal plasma composition varied between ejaculates of the same male; notably, zinc was positively

correlated with the proportion of Y-CBS. Zinc is a major antioxidant. Thus, correlation with Y-CBS is potentially a protective mechanism as Y-CBS are more susceptible to oxidative damage. Fourth, I investigated how variation in sperm sex ratios relative to mating frequency corresponded to variation in foal sex ratios relative to mating frequency. To test the hypothesis that an increase in mating frequency increases the likelihood of daughters, based on my previous results, I first examined foal sex ratios in a population of feral horses consisting of 198 foals sired by 26 feral stallions. More female foals were born when there were more mares available for stallions to mate at the time of conception, linking a high stallion mating frequency to an increase in daughters. I next obtained 46,486 foaling records from 125 commercially breeding stallions in the New Zealand Thoroughbred Racehorse Studbook. Stallion mating frequency did not predict foal sex, suggesting that mating frequency in managed thoroughbreds is not a driver of foal sex ratio skews. The mixed results indicated paternal sex ratio adjustment in response to mating frequency is complex and contextual, potentially relating to variation in sexual rest days prior to mating or the sexual familiarity versus novelty across multiple mating events. Collectively, these results indicate that the proximal modification of sperm and offspring sex ratios is feasible, thereby demonstrating potential paternal control of sex ratios. In this thesis, I have demonstrated that offspring sex ratios are influenced by the degree of prenatal feminisation of maternal physiology. Moreover, I have shown variation in paternal contributions to sex ratios both between and within males, indicating the need for fathers to also be considered in tests of maternal sex allocation. These results suggest that both factors could explain some of the inconsistencies between observation and theory in mammalian sex allocation research and should be accounted for in future sex allocation research.

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General Introduction

Sex allocation theory

Evolutionary theory predicts that individual parents are advantaged by adjusting their allocation of resources into sons and daughters to maximise their inclusive fitness (e.g. Charnov, 1982; Clark, 1978; Fisher, 1930; Hamilton, 1967; Trivers & Willard, 1973). In species with two distinct sexes, this differential allocation includes adjusting the proportions of sons and daughters produced (reviewed in West, 2009). Initial theoretical exploration of sex ratios suggested that biased sex ratios should be rare, as frequency-dependent selection maintains the sex ratio near parity (Düsing, 1884, cited in Edwards, 2000; Darwin 1871; Fisher, 1930). Subsequent theoretical work demonstrated how selection could favour individual parents that conditionally adjust offspring sex ratios to enhance their inclusive fitness (e.g. Charnov *et al.*, 1981; Clark, 1978; Hamilton, 1967; Trivers & Willard, 1973). Several competing, but not mutually exclusive, hypotheses describe parent-specific conditions that differentially affect the fitness returns from sons and daughters and thus, predict adaptive shifts in offspring sex ratios (see Table 1.1) (for reviews, see Hardy, 1997; Komdeur, 2012; Navara, 2018; West, 2009).

Table 1.1. Summary of some of the key adaptive hypotheses used to predict whether parents should produce sons or daughters in vertebrates, particularly those key to mammalian sex allocation.

Hypothesis	*Main assumptions and conditions*	*Main predictions*
'Trivers-Willard' *(Trivers & Willard, 1973)*	- Reproductive success is more variable for one sex (e.g. polygynous species) - Mothers in better condition are able to invest more in offspring - Greater maternal investment confers a competitive advantage to offspring into adulthood - Differences in maternal investment have a greater impact on the more reproductively variable sex than the more stable one	- Mothers in better condition are more likely to produce sons - Mothers in poorer condition are more likely to produce daughters
'Local Resource Competition' *(Clark, 1978)*	- Sex-specific competition within small groups of related individuals for limited resources - Offspring of one sex remain in natal group - Offspring of the other sex disperse	- When competition for resources is higher, mothers should produce more of the dispersing sex
'Local Resource Enhancement' *(Emlen et al., 1986; Gowaty & Lennartz, 1985)*	- Cooperative breeding with helpers - Sons and daughters differ in the assistance they provide to parents in raising the next generation	- Mothers that benefit more from assistance should produce more of the sex that assists
'Advantaged Daughter' *(Altmann, 1980)*	- Female rank is positively associated with reproductive success of daughters - Maternal rank is inherited by philopatric daughters	- Mothers of higher rank should produce more daughters - Mothers of lower rank should produce more sons
'Cost of Reproduction' *(Myers, 1978)*	- The cost of producing one sex is higher than the other - Only mothers in good condition can bear the costs of producing the more costly sex	- Only mothers in better condition should produce the more costly sex - Mothers in poorer condition should produce the cheaper sex
'Male Attractiveness' *(Burley, 1981)*	- Attractive males have higher fitness - Attractive fathers have attractive sons	- Attractive males and their mates should produce more sons - Less attractive males and their mates should produce more daughters

Sex allocation has been generally interpreted in terms of maternal fitness (Table 1.1), with an underlying assumption being that females have the most to gain from adjusting offspring sex ratios (Trivers, 1972). Thus, sex allocation hypotheses have generally focussed on maternal traits that differentially affect the fitness of sons and daughters (see West, 2009). For example, since maternal condition and social rank, and greater competition with philopatric daughters predict differential investment into sons and daughters (Table 1.1), these factors have been extensively investigated as predictors of offspring sex ratios (see Cameron, 2004; Douhard, 2017; Hewison & Gaillard, 1999; Hiraiwa-Hasegawa, 1993; Saltz, 2001; Sheldon & West, 2004; Silk & Brown, 2008). The influence of population density and climatic conditions on maternal traits and subsequently, sex allocation, have also been demonstrated (Blanchard *et al.*, 2005; Kruuk *et al.*, 1999; Moore *et al.*, 2015). Sex ratio skews are also predicted in response to paternal traits, specifically paternal attractiveness (male attractiveness hypothesis, Table 1.1). While theoretical consideration of the male attractiveness hypothesis applies to both mothers and fathers (Burley, 1981), sex ratio skews relative to paternal attractiveness have been primarily interpreted as a maternal response to her mate's attractiveness (see Booksmythe *et al.*, 2017).

A large body of empirical work in a wide variety of taxa indicate that mothers can and do facultatively adjust offspring sex ratios (reviewed in Komdeur, 2012; Navara, 2018; West, 2009). While there have been controversies in sex allocation in vertebrates, particularly mammals, due to inconsistencies in empirical results (see Brown & Silk, 2002; Cameron, 2004; Cockburn *et al.*, 2002; Ewen *et al.*, 2004; Hewison & Gaillard, 1999; Komdeur & Pen, 2002; Krackow, 2002; Sheldon & West, 2004), there are many instances of sex ratios skews in strong accordance with theoretical predictions, implying some degree of maternal control

over sex ratios (e.g. Cameron & Linklater, 2007; Clutton-Brock *et al.*, 1984; Enright *et al.*, 2001; Komdeur *et al.*, 1997; Moore *et al.*, 2015; Rosenfeld *et al.*, 2003; Wauters *et al.*, 1995). An enhanced understanding of sex allocation as it applies to mammalian and other vertebrate mothers has resolved some of the inconsistencies between theory and observation in empirical sex allocation research (e.g. Cameron, 2004; Cameron *et al.*, 2017; Pen & Weissing, 2002; West & Sheldon, 2002). For example, methodological discrepancies in the measurement of predictions and assumptions, including heterogeneous measures of maternal condition, have resolved some of the inconsistencies in tests of the Trivers-Willard hypothesis (Cameron, 2004; Hewison & Gaillard, 1999; Schindler *et al.*, 2015). Furthermore, a better understanding of how the complexities of vertebrate life-histories affect sex allocation, such as trade-offs between current and future reproduction in species with long reproductive lives, has improved sex allocation predictions for vertebrate taxa (e.g. Alonzo & Sheldon, 2010; Moore *et al.*, 2015; Pen & Weissing, 2002; Schindler *et al.*, 2015).

The identification of physiological links between hypothesised cues of sex allocation and offspring sex ratio skews in vertebrates has greatly advanced our understanding of sex allocation in these taxa (for reviews, see Alonso-Alvarez, 2006; Cameron *et al.*, 2017; Geffroy & Douhard, 2019; Navara, 2018; Rutkowska & Badyaev, 2008). Moreover, investigations into the physiological mechanisms of sex ratio adjustment suggest that there are sources of variation in vertebrate sex ratios, particularly in mammals, that have been historically underappreciated (see Douhard, 2017; Edwards *et al.*, 2016b), which might explain some of the remaining inconsistencies in empirical sex allocation research in these taxa (Booksmythe *et al.*, 2017; Komdeur, 2012; Merkling *et al.*, 2018). For example, it has been recently suggested that there may be physiological constraints on maternal sex allocation (Edwards *et*

al., 2016b). Both theoretical and empirical sex allocation research has implicitly assumed equal ability among mothers to facultatively adjust sex ratios to the extent theory predicts (see West, 2009). However, recent evidence indicates that the degree of exposure to key steroid hormones during prenatal development might alter the ability of some mothers to adjust sex ratios (Edwards *et al.*, 2016a; Edwards *et al.*, 2019; Khan *et al.*, 2016; Perret, 2019), potentially constraining sex allocation and producing inconsistencies in empirical results.

Relatively little consideration has also been given to the influence of fathers on sex allocation. In mammals, males are heterogametic and thus, have the opportunity to produce unequal proportions of X- and Y-chromosome-bearing spermatozoa (CBS). Paternal influences on mammalian sex ratios were historically dismissed under the assumption that sperm sex ratios do not vary from parity post-meiosis, however, more recent evidence has challenged this (reviewed by Cameron *et al.*, 2017; Douhard, 2018; Edwards & Cameron, 2014). Thus, paternal influences on sex ratios may be a relatively under-appreciated source of variation in mammalian sex allocation research. In this thesis, I explore these under-appreciated sources of variation in offspring sex ratios: prenatal maternal effects and paternal influences on sex ratios. In this thesis, I focus exclusively on mammals, particularly as mammalian fathers have the opportunity to influence offspring sex ratios through adjustment of sperm sex ratios.

Physiological mechanisms of sex ratio adjustment in mammals

Sex allocation theory in mammals has greatly benefited from recent advances in our understanding of the physiological mechanisms of offspring sex ratio variation at birth (reviewed in Cameron *et al.*, 2017; Navara, 2018; Thurston *et al.*, 2017). Sex ratio adjustment

can occur at three stages: primary sex ratio adjustment, which occurs prior to fertilisation, secondary sex ratio adjustment, which occurs between fertilisation and birth (reviewed in Cameron *et al.*, 2017; Navara & Nelson, 2009), and tertiary sex ratio adjustment, which occurs after birth through, for example, sex-specific parental care (see Veller *et al.*, 2016). In this thesis, I focus on the physiological mechanisms that lead to skews in the offspring sex ratio at birth, which are sex ratios that may be affected by either (or both) primary and secondary sex ratio adjustment. Both primary and secondary sex ratios can shift through several mechanisms occurring throughout reproduction in both males and females (Table 1.2).

Table 1.2. Stages of reproduction at which sex ratio adjustment can occur and physiological hypotheses for adjustment in mammals.

		Mechanism of adjustment	*Hypothesised physiological explanations*
Mating Fertilisation Implantation Gestation	Primary adjustment	*More X- or Y-chromosome-bearing spermatozoa (CBS) ejaculated*	- Sex ratio-distorting genes produce unequal proportions of X- and Y-CBS [1, 2, 3] - Endocrine dysfunction during spermatogenesis/maturation (e.g. from testicular cancer, endocrine-disrupting chemicals, aging) more deleterious to development or viability of X- versus Y-CBS [4, 5, 6, 7, 8, 9] - Greater vulnerability of Y-CBS to oxidative stress exposure [10, 11] during development and maturation
		More X- or Y-CBS reach the oocyte	- Smaller sized Y-CBS have greater motility when seminal or uterine fluid viscosity is higher [12, 13, 14] - Lower survival of Y-CBS when vaginal pH or reactive oxygen species (ROS) elevated [10, 11, 15] - Variation in seminal plasma buffering of vaginal pH or ROS [15, 16, 17] differently affect the viability of X- and Y-CBS [10, 11]
		More X- or Y-CBS able to fertilise the oocyte	- Some spermatogenetic/epididymal conditions (e.g. heat stress, endocrine-disrupting chemicals) differentially affect fertility of X- and Y-CBS [18, 19, 20] - Elevated follicular testosterone during oogenesis increases oocyte receptivity to fertilization by Y-CBS [21, 22, but see 23, 24] - Elevated pre-conceptional glucocorticoids increase oocyte receptivity to fertilization by X-CBS [25, 26]
	Secondary adjustment	*More male or female blastocysts survive and successfully implant*	- High uterine glucose conditions reduce female blastocyst survival and low glucose conditions reduce male survival [27, 28, 29] - Male blastocysts develop more rapidly [30, 31], potentially resulting in skews relating to timing of insemination [32, 33] - Elevated oxidative stress reduces male blastocyst survival [31]
		More male or female embryos and foetuses survive	- Male foetal survival varies with maternal stress and subsequent uterine glucocorticoid concentrations, depending on type and timing of maternal stressors [34, 35, 36, 37, 38, 39]

1. Szyda *et al.*, 2000 2. Van Hooft *et al.*, 2010 3. Stouthamer *et al.*, 2002 4. James, 2006 5. Stone *et al.*, 2013 6. James, 1994 7. Robbins *et al.*, 2008 8. Møller, 1998 9. Song *et al.*, 2018 10. Oyeyipo *et al.*, 2017 11. You *et al.*, 2017 12. Martinez *et al.*, 2004 13. Rohde *et al.*, 1973 14. Martin, 1997 15. Pratt *et al.*, 1987 16. Wai-Sum *et al.*, 2006 17. Poiani, 2006 18. Pérez-Crespo *et al.*, 2008 19. Ishihara *et al.*, 2010 20. You *et al.*, 2018 21. Grant & Irwin, 2005 22. Grant *et al.*, 2008 23. Díez *et al.*, 2009 24. Macaulay *et al.*, 2013 25. Ideta *et al.*, 2009 26. Firman, 2018 27. Green *et al.*, 2016 28. Kimura *et al.*, 2005 29. Larson *et al.*, 2001 30. Ray *et al.*, 1995 31. Pérez-Crespo *et al.*, 2005 32. Krackow *et al.*, 2003 33. Krackow & Burgoyne, 1997 34. Pratt & Lisk, 1989 35. Pratt & Lisk, 1990 36. Navara, 2010 37. Ryan *et al.*, 2011 38. Ryan *et al.*, 2014 39. Linklater, 2007

Biases in sex ratios at birth could hypothetically begin in the male with differential production or maturation of X- and Y-chromosome-bearing spermatozoa (CBS). Indeed, skews in sperm sex ratios have been found to correspond to skews in offspring sex ratios (e.g. Saragusty *et al.*, 2012). Although meiosis will initially produce equal proportions of X- and Y-CBS, spermatogenesis is an extended process (e.g. 34.5 days in mice (*Mus musculus*), Ray *et al.*, 2015; 57 days in horses (*Equus caballus*), Johnson *et al.*, 1997; 74 days in humans, Groswold, 2016). Thus, there is opportunity for sperm sex ratios to deviate from parity between the initial stages of spermatogenesis through to ejaculation (Figure 1.1) (reviewed in Cameron *et al*., 2017). As X- and Y-CBS vary in DNA content, there is variation in size, gene expression, rates of DNA deletions and meiotic errors, and susceptibility to oxidative stress that may lead to differential damage to or survival of X- and Y-CBS in the male (reviewed in Cameron *et al.*, 2017; Rahman & Pang, 2019). For example, sex ratio-distorting genes alter meiotic drive during spermatogenesis to result in unequal representation of X- and Y-CBS (Figure 1.1) (Stouthamer *et al.*, 2002; Van Hooft *et al.*, 2010). Additionally, Y-CBS are more vulnerable to DNA deletions, particularly when induced by elevated oxidative stress (Aitken & Krausz, 2001). Thus, paternal conditions associated with increased oxidative damage, such as advanced age and stress-induced elevations in glucocorticoids (Costantini *et al.*, 2011; Sabeti *et al.*, 2016), may lead to lower proportions of X-CBS represented in the ejaculate if Y-CBS are lost at a greater rate during sperm development and/or maturation (Figure 1.1). In humans, paternal conditions associated with disrupted testosterone production, such as exposure to endocrine-disrupting chemicals, testicular cancer, and aging, are also linked to higher proportions of X-CBS and more daughters (e.g. Astolfi *et al.*, 2001; James, 2008; Martin *et al.*, 1995; Møller, 1998; Robbins *et al.*, 2008), implying impaired testosterone production during sperm development is more deleterious for Y-CBS (Figure 1.1). Moreover, there is evidence

to suggest that natural variation between fathers in testosterone concentrations is linked to variation in offspring sex ratios, with fathers with higher testosterone producing more sons, further supporting the link between paternal testosterone and sperm sex ratios (Gomendio et al., 2006; Perret, 2018; but see Firman et al., 2020).

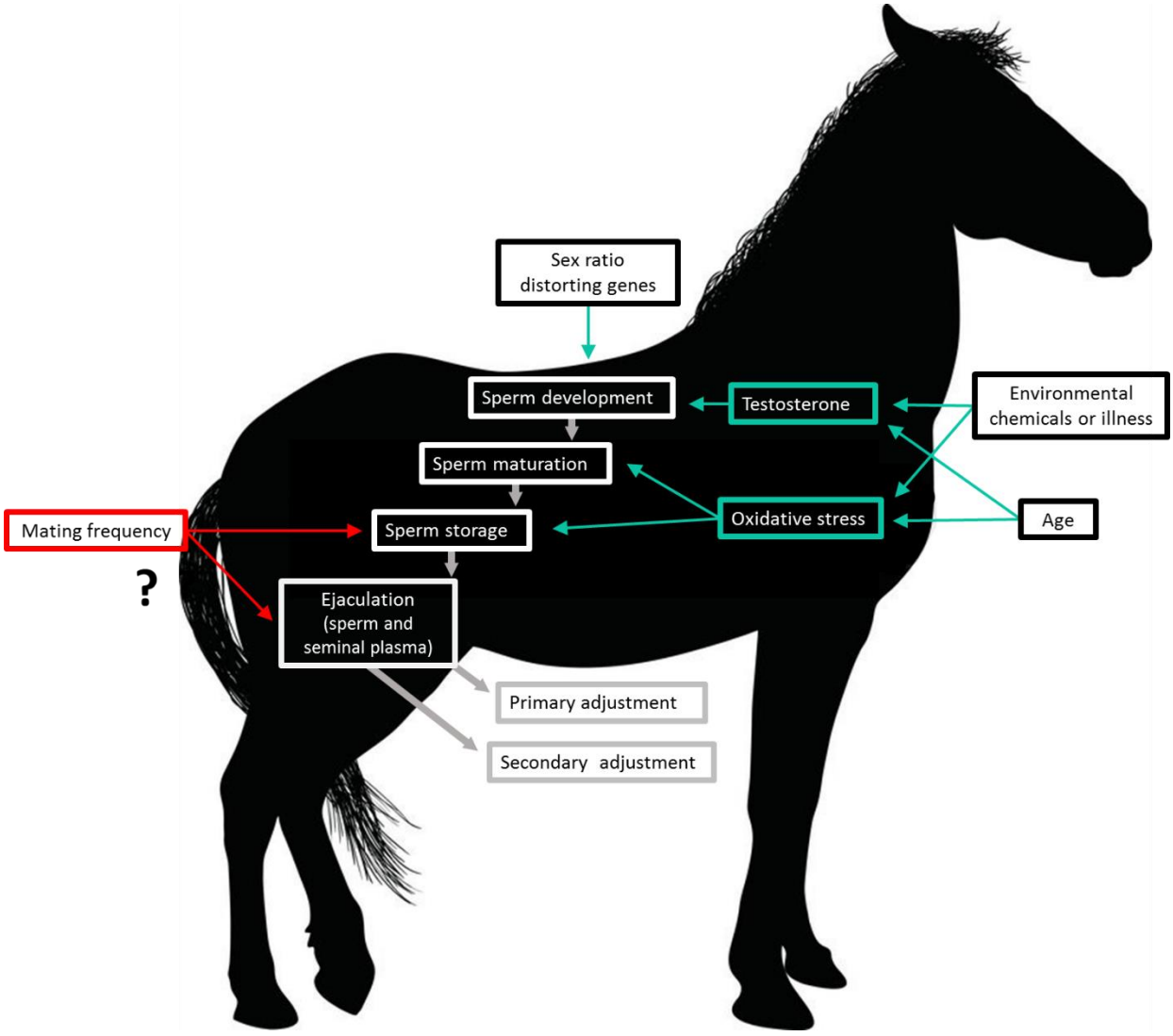

Figure 1.1. Conceptual diagram of the hypothesised physiological pathways (green boxes and arrows) that link paternal factors (black boxes) to skewed sex ratios and the stage during male reproduction that skews most likely arise (white and grey boxes). The majority of research into paternal sex allocation has focused on premating conditions (black boxes) that influence physiological mechanisms (green) during sperm development and maturation. In this thesis, I test whether there are proximal modifiers (red) of sperm sex ratios and seminal plasma composition in the ejaculate that influence offspring sex ratios (grey boxes) by testing whether mating frequency affect components of the ejaculate, potentially through sex-specific effects of sperm storage (red).

Investigations into sperm sex ratio variation to date have primarily focused on premating sources of variation in sperm sex ratios, such as conditions during sperm development and maturation that lead to skewed representations of mature X- and Y-CBS (see Figure 1.1) (for a review, see Edwards & Cameron, 2014). In contrast, whether variation between sperm sex ratios occurs between ejaculates according to proximal breeding conditions has been relatively under-studied. Few studies have examined more than a single ejaculate, despite the considerable inter-ejaculate variation in ejaculate composition (Perry *et al*., 2013). When intra-male variation has been explored, it has been on the basis of two ejaculate samples taken weeks or months apart (e.g. Chandler *et al.*, 2002; DeYoung *et al.*, 2004; Hilsenrath *et al.*, 1997). It is therefore unknown whether ejaculated sperm sex ratios can shift more rapidly in response to a change in the proximal breeding conditions, such as mating frequency (Figure 1.1). Thus, more comprehensive investigations of sperm sex ratios between ejaculates are required, in addition to testing hypothesised mechanisms of potential inter-ejaculate variation. For example, mating frequency could alter sperm sex ratios through variation in the length of sexual rest and hypothesised sex-specific effects during sperm storage (e.g. Olsson *et al*., 2007). Mature spermatozoa are stored in the epididymis prior to ejaculation, during which time they lose viability due to accumulated oxidative damage (Figure 1.1) (Jones, 2004; Pizzari *et al.*, 2008). As rates of oxidative damage differ between the X- and Y-CBS (Oyeyipo *et al.*, 2017; You *et al.*, 2017), extended periods of sexual rest might decrease the proportion of viable Y-CBS in post-sexual rest ejaculates.

After ejaculation, local conditions in the female reproductive tract could modify whether X- or Y-CBS are more likely to arrive at the oocyte, thereby potentially contributing to skews in the sex ratio at fertilisation (see Cameron *et al.,* 2017; Thurston *et al.*, 2017). Recent evidence

suggests the potential for the female oviduct to recognise X- from Y-CBS and respond differently to each (Almiñana *et al.*, 2014), making female primary sex ratio adjustment plausible. The local conditions in the female reproductive tract moderate the passage of spermatozoa on the basis of motility, viability, morphology, gene expression, and potentially chromosome content (X or Y) (reviewed in García-Vázquez *et al.*, 2016; Thurston *et al.*, 2017). For example, human and bovine X- and Y-CBS vary in swimming behaviour and tolerance to pH (Balli *et al.*, 2004; Oyeyipo *et al.*, 2017; Penfold *et al.*, 1998; Sarkar *et al.*, 1984); thus variation in cervical mucus viscosity or vaginal pH could differentially affect the ability of the X- and Y-CBS to traverse the female tract, potentially altering the proportion of X- and Y-CBS arriving at the site of fertilisation (see Table 1.2).

The physiological conditions experienced by spermatozoa in the female reproductive tract are mediated by non-sperm components of the ejaculate, in particular, the seminal plasma (Perry *et al.*, 2013; Robertson & Sharkey, 2016). Therefore, variation in seminal plasma composition and its effects on spermatozoa and/or the female reproductive tract could also potentially influence primary sex ratios (see Cameron *et al.*, 2017). Seminal plasma fulfils a variety of roles that enhance male fertility, including providing energy for spermatozoa and buffering adverse conditions in the female reproductive tract, such as pH and ROS levels (reviewed in Piano, 2006). Thus, sperm competitiveness and fertility are a function of both intrinsic sperm traits and the variation in the composition of seminal plasma (Perry *et al.*, 2013). Seminal plasma composition might differentially support the viability or motility of X- and Y-CBS, thereby skewing the sex ratio of spermatozoa arriving at the site of fertilisation, but this hypothesis that has yet to be empirically tested. Additionally, seminal plasma interacts with the female reproductive tract to influence the developing embryo (Bromfield,

2014; Robertson, 2005) with potentially sex-specific effects (Bromfield *et al.*, 2014). For example, male mice with excised seminal vesicles, which contribute a portion of the seminal plasma, sired sons with altered metabolic function, an effect that was less apparent in their daughters (Bromfield *et al.*, 2014). Several seminal plasma components have hypothesised sex chromosome-specific effects, including testosterone, glucose, and antioxidants (see Table 1.2), but whether these seminal plasma components vary between ejaculates in ways that influence sex allocation in mammals has rarely been tested. A recent study showed that in mice, seminal plasma glucose co-varied with sperm sex ratios, indicating that there is scope for paternal sex allocation to extend beyond fertilisation through seminal plasma composition (Edwards & Cameron, 2017).

After fertilisation, shifts in secondary sex ratios may arise through sex-specific survival of male and female blastocysts relative to the peri-conceptional uterine environment (Table 1.2) (reviewed in Cameron *et al.*, 2017; Rosenfeld, 2011). *Males* and females are sexually dimorphic shortly after fertilisation due to X-linked traits that escape X-inactivation around the time of implantation being expressed more in females than males (Berletch *et al.*, 2011; Carrel & Willard, 2005; Gardner *et al.*, 2010). Thus, prior to implantation, males and females differ in regards to gene expression (Bermejo-Alvarez *et al.*, 2010; Gutierrez-Adan, 2000; Kobayashi *et al.*, 2006) and thus, metabolism (Pérez-Crespo *et al.*, 2005; Sturmey *et al.*, 2010). Consequently, male and female blastocysts vary in their resistance to oxidative stress and survival relative to glucose concentrations, as has been demonstrated *in vitro* (Table 1.2). Thus, secondary sex ratio adjustment might occur through sex-specific embryonic survival according to the peri-conceptional uterine environment (Figure 1.2) (Cameron *et al.*, 2017). Post-implantation embryos continue to show sexual dimorphism throughout gestation as the

upregulation of X-linked traits interacts with maternal stress hormones and nutrition through the placenta to exert sex-specific influences on male and female embryonic survival, leading to skewed offspring sex ratios at birth (reviewed in Rosenfeld, 2011).

The intrauterine environment might also influence sex ratios through trans-generational maternal effects (Clark & Galef, 1995; Fishman *et al.*, 2018). Exposure to testosterone, oestradiol, and glucocorticoids *in utero* can influence the sex ratios a female produces in adulthood (e.g. Dunn *et al.*, 2010; Edwards *et al.*, 2016a). For example, relative exposure to testosterone and oestradiol during prenatal development mediates the process of vertebrate masculinisation and feminisation for males and females (Arnold & McCarthy, 2016; MacLusky & Naftolin, 1981; Morris *et al.*, 2004). In eutherian mammals, differential exposure to prenatal testosterone and oestradiol occurs from variation in endogenous, maternal, placental, and, in litter-bearing species, neighbouring intrauterine sibling sources (Hu *et al.*, 2015; Monclús & Blumstein, 2012; Vom Saal, 1983; Vreeburg *et al.*, 1983). Consequently, both males and females vary in the degree of phenotypic masculinisation and feminisation (Ryan & Vandenbergh, 2002; Vandenbergh, 2003). Phenotypic masculinisation and feminisation affects physiology and reproductive traits, including sex ratios (Ryan & Vandenbergh, 2002; Vandenbergh, 2003). Prenatal masculinisation and feminisation in mice, for example, is linked to variation in glucose metabolism (Roland *et al.*, 2010; Witham *et al.*, 2012) and circulating testosterone (Ryan & Vandenbergh, 2002). As circulating glucose and testosterone are hypothesised to mediate maternal sex allocation (Cameron, 2004; Cameron *et al.*, 2008; Grant, 2007), prenatal masculinisation and feminisation likely affects maternal sex allocation (Figure 1.2). The implications of such prenatal effects on the fitness advantages of sex allocation have been described (Firman, 2020; Fishman *et al.*, 2018; Uller, 2006), but

the potential of physiological constraints has rarely been considered (but see Edwards *et al.*, 2016b).

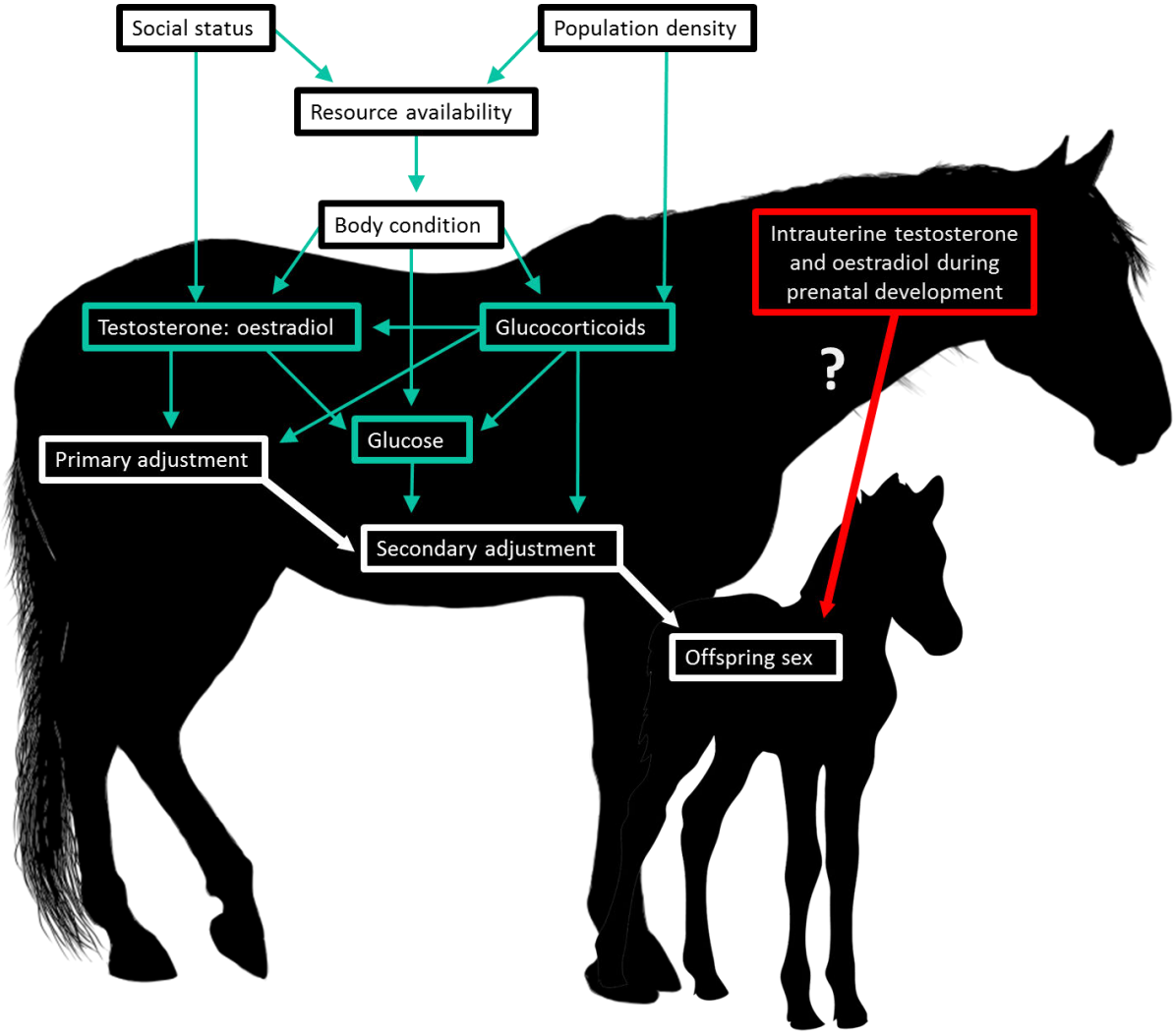

Figure 1.2. Conceptional diagram of how cues of maternal sex allocation (black boxes) likely act through multiple and interacting physiological pathways (green) to influence sex ratios throughout the stages of reproduction (white). Here I test whether intrauterine testosterone and oestradiol exposure during prenatal development (red), affects sex ratio adjustment, potentially presenting constraints on maternal ability to respond to cues of sex allocation.

Links between theoretical and mechanistic hypotheses

Physiological explanations of offspring sex ratios are usually consistent with adaptive predictions (e.g. Cameron, 2004; Cameron *et al.*, 2008), indicating that adaptive maternal control over sex ratios is plausible. For example, the links between maternal body condition, circulating glucose, and the proportion of sons usually corresponds to the predictions of the Trivers-Willard hypothesis (Figure 1.2) (Cameron, 2004). Moreover, increased understanding of the physiological mechanisms of sex ratio adjustment has indicated when adjustment is most likely to occur (see Figure 1.2) and helped to explain some of the inconsistent results of empirical tests of sex allocation (e.g. Cameron, 2004; Cameron *et al.*, 2008; Geffroy & Douhard, 2019; Grant, 2007). For example, when maternal condition as it directly relates to circulating maternal blood glucose (i.e. body condition, body weight, and food intake) is measured near the time of conception, support for the Trivers-Willard hypothesis is over 70% (Cameron, 2004). This timing corresponds to when preimplantation embryos show dimorphic responses to peri-conceptional glucose (Kimura et al., 2005; Larson et al., 2001). This timing is also consistent with predictions that adaptive sex ratio adjustment should occur early in reproduction, as mechanisms that operate close to conception are less costly to females (Almiñana *et al.*, 2014; Fishman, 2018). Conversely, when all studies citing measures of maternal condition (e.g. habitat quality and social status) at various stages throughout gestation are considered, support drops to 34% (Cameron, 2004). Additionally, many of the physiological processes linked to sex ratio skews are interconnected (Figure 1.2) (e.g. Helle *et al.*, 2008; Linklater, 2007; Ryan *et al.*, 2014), potentially explain the sometimes inconsistent results of tests of sex allocation (see Grant, 2007). For example, maternal circulating glucose increases in response to elevations in glucocorticoid concentrations (Manelli & Giustina,

2000; Sapolsky *et al.*, 2000; Viau, 2002) in addition to diet (Figure 1.2), thereby providing explanations for the interactive links between maternal condition, population density, and offspring sex ratios (Kruuk *et al.*, 1999; Moore *et al.*, 2015); maternal glucocorticoid concentrations vary with population density (Creel *et al*., 2013; Dantzer *et al*., 2013).

The physiology of paternal sex allocation has been relatively understudied in comparison to mothers (see Douhard *et al.*, 2018; Edwards *et al.,* 2014). Many of the studies showing skews in offspring sex ratios relative to paternal traits are consistent with theoretical predictions of sex allocation (e.g. Douhard *et al.*, 2016; Gomendio *et al.*, 2006; Perret, 2018; Røed *et al.*, 2007). For example, male red deer (*Cervus elaphus*) with higher fertility and a greater proportion of morphologically normal spermatozoa produce more sons (Gomendio *et al.*, 2006). Since some fertility traits, including sperm morphology, are heritable (Smital *et al.*, 2005), having sons that inherit these traits would enhance the fitness of fathers with higher fertility (male fertility hypothesis; Gomendio *et al.*, 2006). The adaptive significance of sex ratio skews relative to paternal traits requires further empirical investigation, but Douhard *et al.* (2016) demonstrated that paternal reproductive success in male bighorn sheep (*Ovis canadensis*) correlated with the proportion of sons. Since paternal reproductive success reduced the fitness of daughters but increased the average annual success of sons, independent to maternal allocation, paternal sex allocation is implicated (Douhard *et al.*, 2016).

The male fertility hypothesis predicts paternal adjustment of sperm sex ratios in response to heritable traits that influence male reproductive success (Gomendio *et al.*, 2006; Malo *et al*., 2017). More recently it has been suggested that sperm sex ratios are also sensitive to the

social environment, suggesting paternal sex allocation cues may include non-genetic factors as well (Edwards *et al*., 2017; Firman *et al*., 2020; Lavoie *et al*., 2019). For example, male mice exposed to higher levels of male mate competition during post-natal sexual development produced higher proportions of X-CBS, suggesting that fathers adaptively adjust sperm sex ratios according to the intensity of local mate competition (Firman *et al*., 2020). The results of Firman *et al*. (2020) implicated physiological processes influencing spermatogenesis rather than at the time of ejaculation. It remains unknown if fathers also proximately modify the sperm sex ratios between ejaculates according to the current mating environment, but proximal modification of other ejaculate traits, such as sperm numbers per ejaculate, have been well-demonstrated (Burger *et al.*, 2015b; Ferkin, 2004; Jeannerat *et al.*, 2018; Joseph *et al.*, 2015; Lemaitre *et al.*, 2012; Ramm *et al.*, 2015).

Thesis aims and structure

In this thesis, I aim to provide answers to some of the outstanding questions in mammalian sex allocation (Figure 1.3). First, as prenatal effects could impose constraints on maternal sex allocation, there is a need to better understand prenatal influences on sex ratios and how to account for these effects (see Edwards *et al.*, 2016; Fishman *et al*., 2018). Moreover, the understanding of how and when mammalian fathers influence sex ratios remains lacking. Research into paternal sex allocation to date has focused on paternal conditions prior to mating that affect the production of X- and Y-CBS, underpinning population and individual level variation in sperm sex ratios (e.g. Lavoie *et al.*, 2019; Malo *et al.*, 2017; Saragusty *et al.*, 2012; Tiido *et al.*, 2005). Comparatively, there has been little investigation into proximal modifiers of sperm sex ratios that lead to variation at the ejaculate level (but see Chandler *et*

al., 2002; DeYoung *et al*., 2004; Hilsenrath *et al*., 1997). Additionally, investigations into paternal influences on sex ratios have primarily focused on the proportion of X- and Y-CBS, with little consideration of seminal plasma (see Cameron *et al.*, 2017). Thus, there is likely under-appreciated sources of variation in paternal influences on sex ratios.

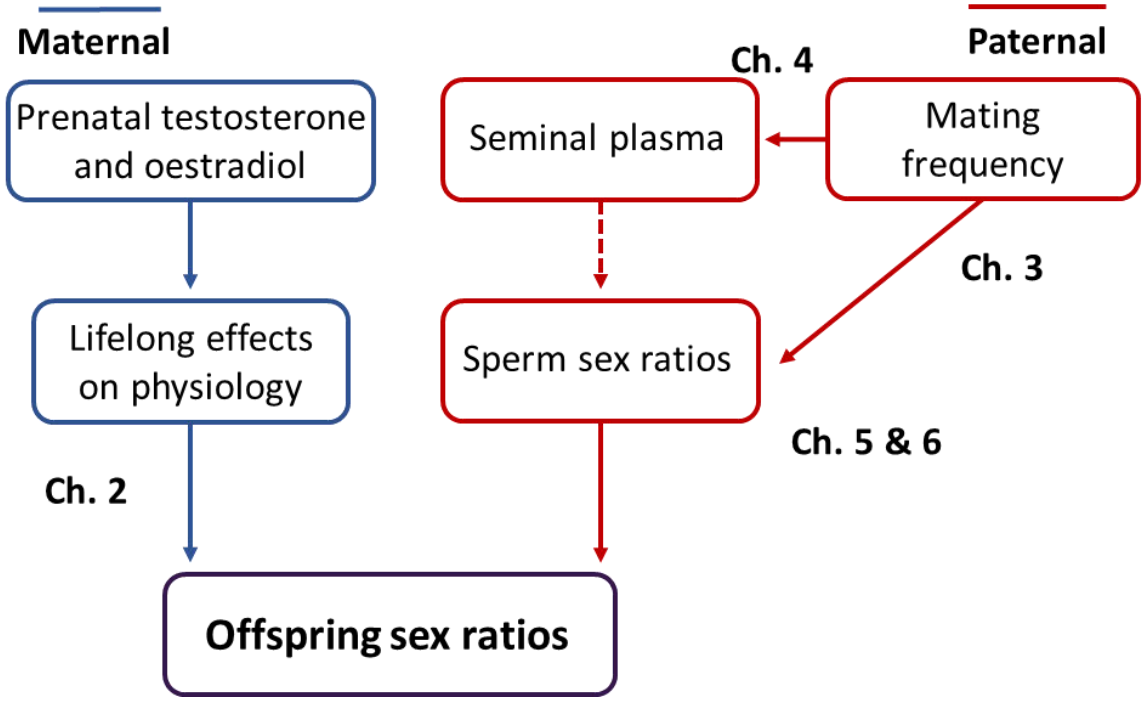

Figure 1.3. Schematic representation of the aims of this thesis.

I first test how prenatal masculinisation and feminisation of female phenotypes in mice (*Mus musculus*) influence later offspring sex ratios (Figure 1.3; Chapter 2). As a litter-bearing species, mice phenotypes are influenced by prenatal testosterone and oestradiol from endogenous, maternal, and placental sources, as well as neighbouring siblings *in utero* (Hu *et al.*, 2015; Monclús & Blumstein, 2012; Vom Saal, 1983; Vreeburg *et al.*, 1983). Thus, there is

variation among individual females in their prenatal testosterone and oestradiol exposure, providing an opportunity to measure the litter sex ratios produced by a range of masculinised to feminised phenotypes in a laboratory setting. Moreover, there are morphological measures in mice that inform the degree to which an individual mouse is masculinised or feminised (Huber *et al.*, 2017; Zheng & Cohn, 2011), which provides a marker for the prenatal environment without the use of invasive techniques such as intrauterine fluid sampling. Moreover, the rapid generation times allow for easier investigation of transgenerational influences, all of which make mice an ideal candidate for studying prenatal influences on maternal sex allocation. I hypothesise that the extent of masculinisation and feminisation will alter litter sex ratios in that more masculinised mothers would produce more sons and more feminised mothers would produce more daughters.

I addressed some of the deficiencies in our understanding of how and when fathers influence sex ratios; specifically, the need to test whether there is within-male variation in sperm sex ratios and seminal plasma composition that might link to paternal control over sex ratios. To examine paternal influences on sex allocation, the domestic horse (*Equus ferus caballus*) was selected as a study species because, firstly, sex allocation relative to maternal traits have been well documented (Cameron & Linklater, 2000, 2007; Cameron *et al.*, 1999; Monard *et al.*, 1997) but the contribution of stallion traits to sex ratio variation has seldom been examined (but see Santos *et al.*, 2015). Secondly, there is considerable variation in ejaculate composition relative to the proximal breeding environment in stallions, including sperm numbers per ejaculate and seminal plasma composition (Burger *et al.*, 2015a; Burger *et al.*, 2015b; Jeannerat *et al.*, 2018), indicating that there is potential for proximal modifiers of ejacualte sperm sex ratios. Thirdly, stallion ejaculates can be easily collected in a domestic

setting and are large in volume, so that a single ejaculate can be subsetted for multiple analyses. And lastly, stallions can be collected repeatedly each day (McKinnon *et al.*, 2011), providing an opportunity to test the effects of an increase in mating frequency on each subsequent ejaculate. Thus, intra-stallion variation in sperm sex ratios and seminal plasma composition can be tested. Moreover, paternal influences on sex ratios can be tested in both a domestic and wild setting using populations of domestic and feral horses. I manipulated ejaculation frequency to test for paternal influences on sex ratios (Figure 1.3). Mating frequency is an important determinant of male reproductive success (Arnold & Duvall, 1994; Preston *et al.*, 2001), potentially providing a cue of male attractiveness (see Edwards *et al.,* 2017). Furthermore, ejaculation frequency affects the duration of time spermatozoa are exposed to hypothesised sex-specific conditions during epididymal storage (see Oyeyipo *et al.*, 2017; You *et al.*, 2017), providing a plausible physiological link between ejaculation frequency and sperm sex ratio skews.

I first test whether sperm sex ratios and seminal plasma components with hypothesised sex-specific effects vary between within-male successive ejaculates (Figure 1.3; Chapter 3 & 4). I test whether ejaculated sperm sex ratios vary at an individual and ejaculate level in two populations of stallions that differed in location, breed, and seasonal ejaculation frequency (Chapter 3). I increase the mating frequency of all stallions to induce sexual exhaustion over three days and measure sperm sex ratios in subsequent ejaculates. I next measure key components of seminal plasma: sex hormones, glucose, major cations and trace elements, and their relationship to X- and Y-CBS in response to sexual exhaustion in one of the previously tested populations of stallions (Chapter 4).

I then test for links between proximal modifiers of sperm sex ratios and offspring sex ratios by testing the relationship between stallion mating frequency and foal sex ratio (Figure 1.3; Chapter 5 & 6). I first test this relationship in a population of feral horses with a dataset consisting of 198 foals sired by 26 stallions in North Dakota, USA (Chapter 5). I measure whether mating frequency during the week of conception as measured by the number of mares available for breeding in a band predicted the sex ratio of a stallion's foals. I assess this relationship next with 46,486 foaling records from 125 racehorse stallions in the New Zealand Thoroughbred Racehorse Studbook (Chapter 6). These racehorse stallions mate at exceptionally high mating frequencies compared to feral stallions, thus allowing me to test the effects of a very high mating frequency on foal sex ratios. I assess the effects of daily, weekly, and seasonal stallion mating frequencies on the probability of siring a son or daughter.

In my general discussion (Chapter 7), I synthesize the findings of my empirical chapters to demonstrate how my results improve our understanding of how and when mammals adjust sex ratios. In particular, my results highlight that complexities in the physiology of mammalian sex ratios adjustment have not been fully addressed in sex allocation research.

References

Aitken, R. J., & Krausz, C. (2001). Oxidative stress, DNA damage and the Y chromosome. *Reproduction, 122*, 497-506.

Almiñana, C., Caballero, I., Heath, P. R., Maleki-Dizaji, S., Parrilla, I., Cuello, C., Gil, M. A., Vazquez, J. L., Vazquez, J. M., & Roca, J. (2014). The battle of the sexes starts in the oviduct: modulation of oviductal transcriptome by X and Y-bearing spermatozoa. *BMC Genomics, 15*, 293.

Alonso-Alvarez, C. (2006). Manipulation of primary sex-ratio: an updated review. *Avian and Poultry Biology Reviews, 17*, 1-20.

Alonzo, S. H., & Sheldon, B. C. (2010). Population density, social behaviour and sex allocation. In T. S., A. M. & J. K. (Eds.) *Social Behaviour: Genes, Ecology, and Evolution* (pp. 474-488). Cambridge, UK: Cambridge University Press.

Altmann, J. (1980). *Baboon Mothers and Infants*. Cambridge, USA: Harvard University Press.

Arnold, A. P., & McCarthy, M. M. (2016). Sexual differentiation of the brain and behavior: a primer. In P. D. & V. N. (Eds.), *Neuroscience in the 21st Century* (pp. 2139-2168). New York, USA: Springer.

Arnold, S. J., & Duvall, D. (1994). Animal mating systems: a synthesis based on selection theory. *The American Naturalist, 143*, 317-348.

Astolfi, P., Cuccia, M., & Martinetti, M. (2001). Paternal HLA genotype and offspring sex ratio. *Human Biology, 73*, 315-319.

Balli, K. S., Patton, W. C., Jacobson, J. D., Corselli, J., King, A., & Chan, P. J. (2004). Sperm velocity in seminal plasma and the association with gender of offspring. *Archives of Andrology, 50*, 37-40.

Bateman, A. J. (1948). Intra-sexual selection in Drosophila. *Heredity, 2*, 349-368.

Berletch, J. B., Yang, F., Xu, J., Carrel, L., & Disteche, C. M. (2011). Genes that escape from X inactivation. *Human Genetics, 130*, 237-245.

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., & Gutierrez-Adan, A. (2010). Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proceedings of the National Academy of Sciences, 107*, 3394-3399.

Blanchard, P., Festa-Bianchet, M., Gaillard, J.-M., & Jorgenson, J. T. (2005). Maternal condition and offspring sex ratio in polygynous ungulates: a case study of bighorn sheep. *Behavioral Ecology, 16*, 274-279.

Booksmythe, I., Mautz, B., Davis, J., Nakagawa, S., & Jennions, M. D. (2017). Facultative adjustment of the offspring sex ratio and male attractiveness: a systematic review and meta-analysis. *Biological Reviews, 92*, 108-134.

Bromfield, J. J. (2014). Seminal plasma and reproduction: much more than previously thought. *Journal of Assisted Reproduction and Genetics, 31*, 627-636.

Bromfield, J. J., Schjenken, J. E., Chin, P. Y., Care, A. S., Jasper, M. J., & Robertson, S. A. (2014). Maternal tract factors contribute to paternal seminal plasma impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences, 111*, 2200-2205.

Brown, G. R., & Silk, J. B. (2002). Reconsidering the null hypothesis: is maternal rank associated with birth sex ratios in primate groups? *Proceedings of the National Academy of Sciences, 99*, 11252-11255.

Burley, N. (1981). Sex ratio manipulation and selection for attractiveness. *Science, 211*, 721-722.

Burger, D., Dolivo, G., Marti, E., Sieme, H., & Wedekind, C. (2015a). Female major histocompatibility complex type affects male testosterone levels and sperm number in the horse (*Equus caballus*). *Proceedings of the Royal Society B: Biological Sciences, 282*, 20150407.

Burger, D., Dolivo, G., & Wedekind, C. (2015b). Ejaculate characteristics depend on social environment in the horse (*Equus caballus*). *PLoS One, 10*, e0143185.

Cameron, E. Z. (2004). Facultative adjustment of mammalian sex ratios in support of the Trivers–Willard hypothesis: evidence for a mechanism. *Proceedings of the Royal Society B: Biological Sciences, 271*, 1723-1728.

Cameron, E. Z., Edwards, A. M., & Parsley, L. M. (2017). Developmental sexual dimorphism and the evolution of mechanisms for adjustment of sex ratios in mammals. *Annals of the New York Academy of Sciences, 1389*, 147-163.

Cameron, E. Z., Lemons, P. R., Bateman, P. W., & Bennett, N. C. (2008). Experimental alteration of litter sex ratios in a mammal. *Proceedings of the Royal Society B: Biological Sciences, 275*, 323-327.

Cameron, E. Z., & Linklater, W. L. (2000). Individual mares bias investment in sons and daughters in relation to their condition. *Animal Behaviour, 60*, 359-367.

Cameron, E. Z., & Linklater, W. L. (2007). Extreme sex ratio variation in relation to change in condition around conception. *Biology Letters, 3*, 395-397.

Cameron, E. Z., Linklater, W. L., Stafford, K. J., & Veltman, C. J. (1999). Birth sex ratios relate to mare condition at conception in Kaimanawa horses. *Behavioral Ecology, 10*, 472-475.

Carrel, L., & Willard, H. F. (2005). X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature, 434*, 400-404.

Chandler, J. E., Canal, A. M., Paul, J. B., & Moser, E. B. (2002). Collection frequency affects percent Y-chromosome-bearing sperm, sperm head area and quality of bovine ejaculates. *Theriogenology, 57*, 1327-1346.

Charnov, E. L., Los-den Hartogh, R., Jones, W., & van den Assem, J. (1981). Sex ratio evolution in a variable environment. *Nature, 289*, 27-32.

Clark, A. B. (1978). Sex ratio and local resource competition in a prosimian primate. *Science, 201*, 163-165.

Clark, M. M., & Galef, B. G. (1995). A gerbil dam's fetal intrauterine position affects the sex ratios of litters she gestates. *Physiology & Behavior, 57*, 297-299.

Clutton-Brock, T. H., Albon, S. D., & Guinness, F. E. (1984). Maternal dominance, breeding success and birth sex ratios in red deer. *Nature, 308*, 358-360.

Cockburn, A., Legge, S., & Double, M. C. (2002). Sex ratios in birds and mammals: can the hypotheses be disentangled. In I. C. Hardy. (Ed.), *Sex Ratios: Concepts and Research Methods* (pp. 266-286). Cambridge, UK: Cambridge University Press.

Costantini, D., Marasco, V., & Møller, A. P. (2011). A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. *Journal of Comparative Physiology B, 181*, 447-456.

Creel, S., Dantzer, B., Goymann, W., & Rubenstein, D. R. (2013). The ecology of stress: effects of the social environment. *Functional Ecology*, *27*, 66-80.

Dantzer, B., Newman, A. E., Boonstra, R., Palme, R., Boutin, S., Humphries, M. M., & McAdam, A. G. (2013). Density triggers maternal hormones that increase adaptive offspring growth in a wild mammal. *Science*, *340*, 1215-1217.

DeYoung, R. W., Muller, L. I., Demarais, S., Guthrie, H. D., Welch, G. R., Engelken, T. J., & Gonzales, R. A. (2004). Do *Odocoileus virginianus* males produce Y-chromosome-biased ejaculates? Implications for adaptive sex ratio theories. *Journal of Mammalogy, 85*, 768-773.

Díez, C., Bermejo-Alvarez, P., Trigal, B., Caamaño, J. N., Muñoz, M., Molina, I., Gutiérrez-Adán, A., Carrocera, S., Martín, D., & Gómez, E. (2009). Changes in testosterone or temperature during the *in vitro* oocyte culture do not alter the sex ratio of bovine embryos. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 311*, 448-452.

Douhard, M. (2017). Offspring sex ratio in mammals and the Trivers-Willard hypothesis: in pursuit of unambiguous evidence. *BioEssays, 39*, 1700043.

Douhard, M. (2018). The role of fathers in mammalian sex allocation. *Mammal Review, 48*, 67-74.

Douhard, M., Festa-Bianchet, M., Coltman, D. W., & Pelletier, F. (2016). Paternal reproductive success drives sex allocation in a wild mammal. *Evolution, 70*, 358-368.

Dunn, E., Kapoor, A., Leen, J., & Matthews, S. G. (2010). Prenatal synthetic glucocorticoid exposure alters hypothalamic–pituitary–adrenal regulation and pregnancy outcomes in mature female guinea pigs. *Journal of Physiology, 588*, 887-899.

Edwards, A. (2000). Carl Düsing (1884) on the regulation of the sex-ratio. *Theoretical Population Biology, 58*, 255-257.

Edwards, A. M., & Cameron, E. Z. (2014). Forgotten fathers: paternal influences on mammalian sex allocation. *Trends in Ecology & Evolution, 29*, 158-164.

Edwards, A. M., & Cameron, E. Z. (2017). Cryptic male choice: experimental evidence of sperm sex ratio and seminal plasma adjustment in relation to coital rate. *Reproduction, Fertility and Development, 29*, 1401-1404.

Edwards, A. M., Cameron, E. Z., Pereira, J. C., Wapstra, E., Ferguson-Smith, M. A., Horton, S. R., & Thomasson, K. (2016a). Gestational experience alters sex allocation in the subsequent generation. *Royal Society Open Science, 3*, 160210.

Edwards, A. M., Cameron, E. Z., & Wapstra, E. (2016b). Are there physiological constraints on maternal ability to adjust sex ratios in mammals? *Journal of Zoology, 299*, 1-9.

Edwards, A. M., Cameron, E. Z., Wapstra, E., & McEvoy, J. (2019). Maternal effects obscure condition-dependent sex allocation in changing environments. *Royal Society Open Science, 6*, 181885.

Emlen, S. T., Emlen, J. M., & Levin, S. A. (1986). Sex-ratio selection in species with helpers-at-the-nest. *The American Naturalist, 127*, 1-8.

Enright, W., Spicer, L., Kelly, M., Culleton, N., & Prendiville, D. (2001). Energy level in winter diets of fallow deer: effect on plasma levels of insulin-like growth factor-I and sex ratio of their offspring. *Small Ruminant Research, 39*, 253-259.

Ewen, J. G., Cassey, P., & Møller, A. P. (2004). Facultative primary sex ratio variation: a lack of evidence in birds? *Proceedings of the Royal Society B: Biological Sciences, 271*, 1277-1282.

Ferkin, M. H. (2004). Male mammals respond to a risk of sperm competition conveyed by odours of conspecific males. *Nature, 431*, 446-449.

Firman, R. C. (2020). Exposure to high male density causes maternal stress and female-biased sex ratios in a mammal. *Proceedings of the Royal Society B: Biological Sciences*, *287*, 20192909.

Firman, R. C., Tedeschi, J. N., & Garcia-Gonzalez, F. (2020). Sperm sex ratio adjustment in a mammal: perceived male competition leads to elevated proportions of female-producing sperm. *Biology Letters, 16*, 20190929.

Fisher, R. A. (1930). *The Genetical Theory of Natural Selection*. Oxford, UK: Oxford University Press.

Fishman, R., Vortman, Y., Shanas, U., & Koren, L. (2018). Cortisol advantage of neighbouring the opposite sex *in utero*. *Royal Society open science, 5*, 171636.

García-Vázquez, F. A., Gadea, J., Matás, C., & Holt, W. V. (2016). Importance of sperm morphology during sperm transport and fertilization in mammals. *Asian Journal of Andrology, 18*, 844-850.

Gardner, D. K., Larman, M. G., & Thouas, G. A. (2010). Sex-related physiology of the preimplantation embryo. *MHR: Basic Science of Reproductive Medicine, 16*, 539-547.

Geffroy, B., & Douhard, M. (2019). The adaptive sex in stressful environments. *Trends in Ecology & Evolution, 34*, 628-640.

Gomendio, M., Malo, A. F., Soler, A. J., Fernández-Santos, M. R., Esteso, M. C., García, A. J., Roldan, E. R., & Garde, J. (2006). Male fertility and sex ratio at birth in red deer. *Science, 314*, 1445-1447.

Gowaty, P. A., & Lennartz, M. R. (1985). Sex ratios of nestling and fledgling red-cockaded woodpeckers (*Picoides borealis*) favor males. *The American Naturalist, 126*, 347-353.

Grant, V. J. (2007). Could maternal testosterone levels govern mammalian sex ratio deviations? *Journal of Theoretical Biology, 246*, 708-719.

Grant, V. J., & Irwin, R. (2005). Follicular fluid steroid levels and subsequent sex of bovine embryos. *Journal of Experimental Zoology A: Comparative Experimental Biology, 303*, 1120-1125.

Grant, V. J., Irwin, R., Standley, N., Shelling, A., & Chamley, L. (2008). Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. *Biology of Reproduction, 78*, 812-815.

Green, M. P., Harvey, A. J., Spate, L. D., Kimura, K., Thompson, J. G., & Roberts, R. M. (2016). The effects of 2, 4-dinitrophenol and d-glucose concentration on the development, sex ratio, and interferon-tau (IFNT) production of bovine blastocysts. *Molecular Reproduction and Development, 83*, 50-60.

Griswold, M. D. (2016). Spermatogenesis: the commitment to meiosis. *Physiological Reviews*, *96*, 1-17.

Gutierrez-Adan, A., Oter, M., Martinez-Madrid, B., Pintado, B., de La Fuente, J. (2000). Differential expression of two genes located on the X chromosome between male and female *in vitro*-produced bovine embryos at the blastocyst stage. *Molecular Reproduction and Development, 55*, 146-151.

Hamilton, W. D. (1967). Extraordinary sex ratios. *Science, 156*, 477-488.

Hardy, I. C. (1997). Possible factors influencing vertebrate sex ratios: an introductory overview. *Applied Animal Behaviour Science, 51*, 217-241.

Helle, S., Laaksonen, T., Adamsson, A., Paranko, J., & Huitu, O. (2008). Female field voles with high testosterone and glucose levels produce male-biased litters. *Animal Behaviour, 75*, 1031-1039.

Hewison, A. M., & Gaillard, J. M. (1999). Successful sons or advantaged daughters? The Trivers–Willard model and sex-biased maternal investment in ungulates. *Trends in Ecology & Evolution, 14*, 229-234.

Hilsenrath, R. E., Swarup, M., Bischoff, F. Z., Buster, J. E., & Carson, S. A. (1997). Effect of sexual abstinence on the proportion of X-bearing sperm as assessed by multicolor fluorescent *in situ* hybridization. *Fertility and Sterility, 68*, 510-513.

Hiraiwa-Hasegawa, M. (1993). Skewed birth sex ratios in primates: should high-ranking mothers have daughters or sons? *Trends in Ecology & Evolution, 8*, 395-400.

Hu, M., Richard, J. E., Maliqueo, M., Kokosar, M., Fornes, R., Benrick, A., Jansson, T., Ohlsson, C., Wu, X., & Skibicka, K. P. (2015). Maternal testosterone exposure increases anxiety-like behavior and impacts the limbic system in the offspring. *Proceedings of the National Academy of Sciences, 112*, 14348-14353.

Ideta, A., Hayama, K., Kawashima, C., Urakawa, M., Miyamoto, A., & Aoyagi, Y. (2009). Subjecting holstein heifers to stress during the follicular phase following superovulatory treatment may increase the female sex ratio of embryos. *Journal of Reproduction and Development, 55*, 529-533.

Ishihara, K., Ohsako, S., Tasaka, K., Harayama, H., Miyake, M., Warita, K., Tanida, T., Mitsuhashi, T., Nanmori, T., & Tabuchi, Y. (2010). When does the sex ratio of offspring of the paternal 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) exposure decrease: in the spermatozoa stage or at fertilization? *Reproductive Toxicology, 29*, 68-73.

James, W. H. (1994). Sex ratios of the offspring of patients with multiple sclerosis. *Neuroepidemiology, 13*, 216-219.

James, W. H. (2006). Offspring sex ratios at birth as markers of paternal endocrine disruption. *Environmental Research, 100*, 77-85.

James, W. H. (2008). Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Journal of Endocrinology, 198*, 3-15.

Jeannerat, E., Marti, E., Berney, C., Janett, F., Bollwein, H., Sieme, H., Burger, D., & Wedekind, C. (2018). Stallion semen quality depends on major histocompatibility complex matching to teaser mare. *Molecular Ecology, 27*, 1025-1035.

Joseph, P. N., Sharma, R. K., Agarwal, A., & Sirot, L. K. (2015). Men ejaculate larger volumes of semen, more motile sperm, and more quickly when exposed to images of novel women. *Evolutionary Psychological Science, 1*, 195-200.

Johnson, L., Blanchard, T., Varner, D., & Scrutchfield, W. (1997). Factors affecting spermatogenesis in the stallion. *Theriogenology, 48*, 1199-1216.

Jones, R. (2004). Sperm survival versus degradation in the mammalian epididymis: a hypothesis. *Biology of Reproduction, 71*, 1405-1411.

Khan, N., Peters, R. A., Richardson, E., & Robert, K. A. (2016). Maternal corticosterone exposure has transgenerational effects on grand-offspring. *Biology Letters, 12*, 20160627.

Kimura, K., Spate, L., Green, M., & Roberts, R. M. (2005). Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. *Molecular Reproduction and Development, 72*, 201-207.

Kobayashi, S., Isotani, A., Mise, N., Yamamoto, M., Fujihara, Y., Kaseda, K., Nakanishi, T., Ikawa, M., Hamada, H., & Abe, K. (2006). Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. *Current Biology, 16*, 166-172.

Komdeur, J. (2012). Sex allocation. In M. Kölliker (Ed.), *The Evolution of Parental Care* (pp. 171-188). Oxford, UK: OUP Oxford.

Komdeur, J., Daan, S., Tinbergen, J., & Mateman, C. (1997). Extreme adaptive modification in sex ratio of the Seychelles warbler's eggs. *Nature, 385*, 522-525.

Komdeur, J., & Pen, I. (2002). Adaptive sex allocation in birds: the complexities of linking theory and practice. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 357*, 373-380.

Krackow, S. (2002). Why parental sex ratio manipulation is rare in higher vertebrates. *Ethology, 108*, 1041-1056.

Krackow, S., & Burgoyne, P. (1997). Timing of mating, developmental asynchrony and the sex ratio in mice. *Physiology & Behavior, 63*, 81-84.

Krackow, S., Schmidt, T., & Elepfandt, A. (2003). Sexual growth dimorphism affects birth sex ratio in house mice. *Proceedings of the Royal Society B: Biological Sciences, 270*, 943-947.

Kruuk, L. E., Clutton-Brock, T. H., Albon, S. D., Pemberton, J. M., & Guinness, F. E. (1999). Population density affects sex ratio variation in red deer. *Nature, 399*, 459-461.

Larson, M. A., Kimura, K., Kubisch, H. M., & Roberts, R. M. (2001). Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-τ. *Proceedings of the National Academy of Sciences, 98*, 9677-9682.

Lavoie, M. D., Tedeschi, J. N., Garcia-Gonzalez, F., & Firman, R. C. (2019). Exposure to male-dominated environments during development influences sperm sex ratios at sexual maturity. *Evolution Letters, 3*, 392-402.

Lemaitre, J. F., Ramm, S. A., Hurst, J. L., & Stockley, P. (2012). Sperm competition roles and ejaculate investment in a promiscuous mammal. *Journal of Evolutionary Biology, 25*, 1216-1225.

Linklater, W. L. (2007). Translocation reverses birth sex ratio bias depending on its timing during gestation: evidence for the action of two sex-allocation mechanisms. *Reproduction, Fertility and Development, 19*, 831-839.

Macaulay, A. D., Hamilton, C. K., King, W. A., & Bartlewski, P. M. (2013). Influence of physiological concentrations of androgens on the developmental competence and sex ratio of *in vitro* produced bovine embryos. *Reproductive Biology, 13*, 41-50.

MacLusky, N. J., & Naftolin, F. (1981). Sexual differentiation of the central nervous system. *Science, 211*, 1294-1303.

Malo, A. F., Martinez-Pastor, F., Garcia-Gonzalez, F., Garde, J., Ballou, J. D., & Lacy, R. C. (2017). A father effect explains sex-ratio bias. *Proceedings of the Royal Society B: Biological Sciences, 284*, 20171159.

Manelli, F., & Giustina, A. (2000). Glucocorticoid-induced osteoporosis. *Trends in Endocrinology & Metabolism, 11*, 79-85.

Martin, J. F. (1997). Length of the follicular phase, time of insemination, coital rate and the sex of offspring. *Human Reproduction, 12*, 611-616.

Martin, R. H., Spriggs, E., Ko, E., & Rademaker, A. W. (1995). The relationship between paternal age, sex ratios, and aneuploidy frequencies in human sperm, as assessed by multicolor FISH. *American Journal of Human Genetics, 57*, 1395-1399.

Martinez, F., Kaabi, M., Martinez-Pastor, F., Alvarez, M., Anel, E., Boixo, J., De Paz, P., & Anel, L. (2004). Effect of the interval between estrus onset and artificial insemination on sex ratio and fertility in cattle: a field study. *Theriogenology, 62*, 1264-1270.

McKinnon, A. O., Squires, E. L., Vaala, W. E., & Varner, D. D. (2011). *Equine Reproduction*: John Wiley & Sons.

Merkling, T., Nakagawa, S., Lagisz, M., & Schwanz, L. E. (2018). Maternal testosterone and offspring sex-ratio in birds and mammals: a meta-analysis. *Evolutionary Biology, 45*, 96-104.

Møller, H. (1998). Trends in sex-ratio, testicular cancer and male reproductive hazards: are they connected? *Journal of Pathology, Microbiology, and Immunology, 106*, 232-239.

Monard, A. M., Duncan, P., Fritz, H., & Feh, C. (1997). Variations in the birth sex ratio and neonatal mortality in a natural herd of horses. *Behavioral Ecology and Sociobiology, 41*, 243-249.

Monclús, R., & Blumstein, D. T. (2012). Litter sex composition affects life-history traits in yellow-bellied marmots. *Journal of Animal Ecology, 81*, 80-86.

Moore, E. P., Hayward, M., & Robert, K. A. (2015). High density, maternal condition, and stress are associated with male-biased sex allocation in a marsupial. *Journal of Mammalogy, 96*, 1203-1213.

Morris, J. A., Jordan, C. L., & Breedlove, S. M. (2004). Sexual differentiation of the vertebrate nervous system. *Nature Neuroscience, 7*, 1034-1039.

Myers, J. H. (1978). Sex ratio adjustment under food stress: maximization of quality or numbers of offspring? *The American Naturalist, 112*, 381-388.

Navara, K. J. (2010). Programming of offspring sex ratios by maternal stress in humans: assessment of physiological mechanisms using a comparative approach. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 180*, 785-796.

Navara, K. J. (2018). *Choosing Sexes: Mechanisms and Adaptive Patterns of Sex Allocation in Vertebrates*. New York, USA: Springer.

Navara, K. J., & Nelson, R. J. (2009). Prenatal environmental influences on the production of sex-specific traits in mammals. *Seminars in Cell & Developmental Biology, 20*, 313-319.

Olsson, M., Schwartz, T., Uller, T., & Healey, M. (2007). Sons are made from old stores: sperm storage effects on sex ratio in a lizard. *Biology Letters*, *3*, 491-493.

Oyeyipo, I. P., van der Linde, M., & du Plessis, S. S. (2017). Environmental exposure of sperm sex-chromosomes: a gender selection technique. *Toxicological Research, 33*, 315-323.

Pen, I., & Weissing, F. J. (2002). Optimal sex allocation: steps towards a mechanistic theory. In I. C. Hardy (Ed.), *Sex Ratios: Concepts and Research Methods* (pp. 26-45). Cambridge, UK: Cambridge University Press.

Penfold, L., Holt, C., Holt, W., Welch, G., Cran, D., & Johnson, L. (1998). Comparative motility of X and Y chromosome-bearing bovine sperm separated on the basis of DNA content by flow sorting. *Molecular Reproduction and Development: Incorporating Gamete Research, 50*, 323-327.

Pérez-Crespo, M., Pintado, B., & Gutiérrez-Adán, A. (2008). Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Molecular Reproduction and Development, 75*, 40-47.

Pérez-Crespo, M., Ramírez, M., Fernández-González, R., Rizos, D., Lonergan, P., Pintado, B., & Gutiérrez-Adán, A. (2005). Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. *Molecular Reproduction and Development: Incorporating Gamete Research, 72*, 502-510.

Perret, M. (2018). Revisiting the Trivers-Willard theory on birth sex ratio bias: role of paternal condition in a Malagasy primate. *PLoS One, 13*, e0209640.

Perret, M. (2019). Litter sex composition affects first reproduction in female grey mouse lemurs (*Microcebus murinus*). *Physiology & Behavior, 208*, 112575.

Perry, J. C., Sirot, L., & Wigby, S. (2013). The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution, 28*, 414-422.

Pike, T. W., & Petrie, M. (2003). Potential mechanisms of avian sex manipulation. *Biological Reviews, 78*, 553-574.

Pizzari, T., Dean, R., Pacey, A., Moore, H., & Bonsall, M. B. (2008). The evolutionary ecology of pre- and post-meiotic sperm senescence. *Trends in Ecology & Evolution, 23*, 131-140.

Poiani, A. (2006). Complexity of seminal plasma: a review. *Behavioral Ecology and Sociobiology, 60*, 289-310.

Pratt, N. C., Huck, U. W., & Lisk, R. D. (1987). Offspring sex ratio in hamsters is correlated with vaginal pH at certain times of mating. *Behavioral and Neural Biology, 48*, 310-316.

Pratt, N. C., & Lisk, R. D. (1989). Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (*Mesocricetus auratus*). *Reproduction, 87*, 763-769.

Pratt, N. C., & Lisk, R. D. (1990). Dexamethasone can prevent stress-related litter deficits in the golden hamster. *Behavioral and Neural Biology, 54*, 1-12.

Preston, B. T., Stevenson, I. R., Pemberton, J. M., & Wilson, K. (2001). Dominant rams lose out by sperm depletion. *Nature, 409*, 681-682.

Rahman, M. S., & Pang, M.-G. (2019). New biological insights on X and Y chromosome-bearing spermatozoa. *Frontiers in Cell and Developmental Biology, 7*, 1-19.

Ramm, S. A., Edward, D. A., Claydon, A. J., Hammond, D. E., Brownridge, P., Hurst, J. L., Beynon, R. J., & Stockley, P. (2015). Sperm competition risk drives plasticity in seminal plasma composition. *BMC Biology, 13*, 1-18.

Ray, D., Pitts, P. B., Hogarth, C. A., Whitmore, L. S., Griswold, M. D., & Ye, P. (2015). Computer simulations of the mouse spermatogenic cycle. *Biology Open, 4*, 1-12.

Ray, P., Conaghan, J., Winston, R., & Handyside, A. (1995). Increased number of cells and metabolic activity in male human preimplantation embryos following *in vitro* fertilization. *Reproduction, 104*, 165-171.

Reiss, M. J. (1987). Evolutionary conflict over the control of offspring sex ratio. *Journal of Theoretical Biology, 125*, 25-39.

Robbins, W. A., Wei, F., Elashoff, D. A., Wu, G., Xun, L., & Jia, J. (2008). Y: X sperm ratio in boron-exposed men. *Journal of Andrology, 29*, 115-121.

Robertson, S. A. (2005). Seminal plasma and male factor signalling in the female reproductive tract. *Cell and Tissue Research, 322*, 43-52.

Robertson, S. A., & Sharkey, D. J. (2016). Seminal plasma and fertility in women. *Fertility and Sterility, 106*, 511-519.

Røed, K. H., Holand, Ø., Mysterud, A., Tverdal, A., Kumpula, J., & Nieminen, M. (2007). Male phenotypic quality influences offspring sex ratio in a polygynous ungulate. *Proceedings of the Royal Society B: Biological Sciences, 274*, 727-733.

Rohde, W., Porstmann, T., & Doerner, G. (1973). Migration of Y-bearing human spermatozoa in cervical mucus. *Reproduction, 33*, 167-169.

Roland, A. V., Nunemaker, C. S., Keller, S. R., & Moenter, S. M. (2010). Prenatal androgen exposure programs metabolic dysfunction in female mice. *Journal of Endocrinology, 207*, 213-223.

Rosenfeld, C. S. (2011). Periconceptional influences on offspring sex ratio and placental responses. *Reproduction, Fertility and Development, 24*, 45-58.

Rosenfeld, C. S., Grimm, K. M., Livingston, K. A., Brokman, A. M., Lamberson, W. E., & Roberts, R. M. (2003). Striking variation in the sex ratio of pups born to mice according to whether maternal diet is high in fat or carbohydrate. *Proceedings of the National Academy of Sciences, 100*, 4628-4632.

Rutkowska, J., & Badyaev, A. V. (2008). Meiotic drive and sex determination: molecular and cytological mechanisms of sex ratio adjustment in birds. *Philosophical Transactions of the Royal Society B: Biological Sciences, 363*, 1675-1686.

Ryan, B. C., & Vandenbergh, J. G. (2002). Intrauterine position effects. *Neuroscience & Biobehavioral Reviews, 26*, 665-678.

Ryan, C. P., Anderson, W. G., Berkvens, C. N., & Hare, J. F. (2014). Maternal gestational cortisol and testosterone are associated with trade-offs in offspring sex and number in a free-living rodent (*Urocitellus richardsonii*). *PLoS One, 9*, e111052.

Ryan, C. P., Anderson, W. G., Gardiner, L. E., & Hare, J. F. (2011). Stress-induced sex ratios in ground squirrels: support for a mechanistic hypothesis. *Behavioral Ecology, 23*, 160-167.

Sabeti, P., Pourmasumi, S., Rahiminia, T., Akyash, F., & Talebi, A. R. (2016). Etiologies of sperm oxidative stress. *International Journal of Reproductive Biomedicine, 14*, 231-240.

Saltz, D. (2001). Progeny sex ratio variation in ungulates: maternal age meets environmental perturbation of demography. *Oikos, 94*, 377-384.

Santos, M. M., Maia, L. L., Nobre, D. M., Neto, J. F. O., Garcia, T. R., Lage, M. C. G. R., de Melo, M. I. V., Viana, W. S., Palhares, M. S., & da Silva Filho, J. M. (2015). Sex ratio of equine offspring is affected by the ages of the mare and stallion. *Theriogenology, 84*, 1238-1245.

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews, 21*, 55-89.

Saragusty, J., Hermes, R., Hofer, H., Bouts, T., Göritz, F., & Hildebrandt, T. B. (2012). Male pygmy hippopotamus influence offspring sex ratio. *Nature Communications, 3*, 1-5.

Sarkar, S., Jolly, D. J., Friedmann, T., & Jones, O. W. (1984). Swimming behavior of X and Y human sperm. *Differentiation, 27*, 120-125.

Schindler, S., Gaillard, J. M., Grüning, A., Neuhaus, P., Traill, L. W., Tuljapurkar, S., & Coulson, T. (2015). Sex-specific demography and generalization of the Trivers–Willard theory. *Nature, 526*, 249-252.

Sheldon, B. C., & West, S. A. (2004). Maternal dominance, maternal condition, and offspring sex ratio in ungulate mammals. *The American Naturalist, 163*, 40-54.

Silk, J. B., & Brown, G. R. (2008). Local resource competition and local resource enhancement shape primate birth sex ratios. *Proceedings of the Royal Society B: Biological Sciences, 275*, 1761-1765.

Smital, J., Wolf, J., & De Sousa, L. (2005). Estimation of genetic parameters of semen characteristics and reproductive traits in AI boars. *Animal Reproduction Science, 86*, 119-130.

Song, W. H., Mohamed, E. A., Pang, W. K., Kang, K. H., Ryu, D. Y., Rahman, M. S., & Pang, M. G. (2018). Effect of endocrine disruptors on the ratio of X and Y chromosome-bearing live spermatozoa. *Reproductive Toxicology, 82*, 10-17.

Stone, B. A., Alex, A., Werlin, L. B., & Marrs, R. P. (2013). Age thresholds for changes in semen parameters in men. *Fertility and Sterility, 100*, 952-958.

Stouthamer, R., Hurst, G. D., & Breeuwer, J. A. (2002). Sex ratio distorters and their detection. In I. C. Hardy (Ed.), *Sex Ratios. Concepts and Research Methods.* (pp. 195-215). Cambridge, UK: Cambridge University Press.

Sturmey, R., Bermejo-Alvarez, P., Gutierrez-Adan, A., Rizos, D., Leese, H., & Lonergan, P. (2010). Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Molecular Reproduction and Development: Incorporating Gamete Research, 77*, 285-296.

Szyda, J., Simianer, H., & Lien, S. (2000). Sex ratio distortion in bovine sperm correlates to recombination in the pseudoautosomal region. *Genetical Research, 75*, 53-59.

Thurston, L. M., Holt, W. V., & Fazeli, A. (2017). Battle of the sexes: how the selection of spermatozoa in the female reproductive tract manipulates the sex ratio of offspring. In M. Legato (Ed.), *Principles of Gender-Specific Medicine* (pp. 251-267), USA: Academic Press.

Trivers, R. (1972). Parental investment and sexual selection. In *Sexual Selection & the Descent of Man (pp.* 136-179), Chicago, USA: Aldine Publishing Company.

Trivers, R. L., & Willard, D. E. (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science, 179*, 90-92.

Uller, T. (2006). Sex-specific sibling interactions and offspring fitness in vertebrates: patterns and implications for maternal sex ratios. *Biological Reviews, 81*, 207-217.

Van Hooft, P., Prins, H. H., Getz, W. M., Jolles, A. E., van Wieren, S. E., Greyling, B. J., van Helden, P. D., & Bastos, A. D. (2010). Rainfall-driven sex-ratio genes in African buffalo suggested by correlations between Y-chromosomal haplotype frequencies and foetal sex ratio. *BMC Evolutionary Biology, 10*, 1-11.

Vandenbergh, J. G. (2003). Prenatal hormone exposure and sexual variation: hormone exposure in the womb is instrumental in shaping the sexual anatomy, physiology and behavior of mammals. *American Scientist, 91*, 218-225.

Veller, C., Haig, D., & Nowak, M. A. (2016). The Trivers–Willard hypothesis: sex ratio or investment? *Proceedings of the Royal Society B: Biological Sciences, 283*, 20160126.

Viau, V. (2002). Functional cross-talk between the hypothalamic-pituitary-gonadal and-adrenal axes. *Journal of Neuroendocrinology, 14*, 506-513.

Vom Saal, F. S. (1983). The interaction of circulating oestrogens and androgens in regulating mammalian sexual differentiation. In B. J., P. E., & G. R. (Eds.), *Hormones and Behaviour in Higher Vertebrates* (pp. 159-177). Berlin, Germany: Springer-Verlag.

Vreeburg, J., Groeneveld, J., Post, P., & Ooms, M. (1983). Concentrations of testosterone and androsterone in peripheral and umbilical venous plasma of fetal rats. *Journal of Reproduction and Fertility, 68*, 171-175.

Wai-Sum, O., Chen, H., & Chow, P. (2006). Male genital tract antioxidant enzymes - their ability to preserve sperm DNA integrity. *Molecular and Cellular Endocrinology, 250*, 80-83.

Wauters, L. A., de Crombrugghe, S. A., Nour, N., & Matthysen, E. (1995). Do female roe deer in good condition produce more sons than daughters. *Behavioral Ecology and Sociobiology, 37*, 189-193.

West, S. (2009). *Sex Allocation*. Princeton, USA: Princeton University Press.

West, S. A., & Sheldon, B. C. (2002). Constraints in the evolution of sex ratio adjustment. *Science, 295*, 1685-1688.

Williams, G. (1979). The question of adaptive sex ratio in outcrossed vertebrates. *Proceedings of the Royal Society B: Biological Sciences, 205*, 567-580.

Witham, E. A., Meadows, J. D., Shojaei, S., Kauffman, A. S., & Mellon, P. L. (2012). Prenatal exposure to low levels of androgen accelerates female puberty onset and reproductive senescence in mice. *Endocrinology, 153*, 4522-4532.

You, Y. A., Mohamed, E. A., Rahman, M. S., Kwon, W. S., Song, W. H., Ryu, B. Y., & Pang, M. G. (2018). 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin can alter the sex ratio of embryos with decreased viability of Y spermatozoa in mice. *Reproductive Toxicology, 77*, 130-136.

You, Y. A., Kwon, W. S., Saidur Rahman, M., Park, Y. J., Kim, Y. J., & Pang, M. G. (2017). Sex chromosome-dependent differential viability of human spermatozoa during prolonged incubation. *Human Reproduction, 32*, 1183-1191.

Chapter 2: Prenatal masculinisation and feminisation affects future maternal sex allocation

Abstract

Sex allocation theory predicts that parents would be advantaged by adjusting offspring sex ratios according to the relative fitness returns from sons versus daughters. Inconsistencies between theoretical predictions and empirical results in mammalian sex allocation research suggest that there may be physiological constraints on some females' capacity to adjust sex ratios to the extent theory predicts. Variation in exposure to testosterone and oestradiol during prenatal development may affect future maternal sex allocation due to permanent masculinising and feminising effects on female physiology. I assessed whether the degree of masculinisation or feminisation of female mice (*Mus musculus*) was related to litter sex ratios or exploratory behaviour using the ratio of the second to fourth digit (digit ratio) as the primary measure of masculinisation and feminisation. Females with feminised digit ratios had more daughters. Only directed exploratory behaviour was related to digit ratio, but this behavioural trait did not correlate with litter sex ratios. These results suggest that the degree of phenotypic masculinisation/feminisation influences the litter sex ratios produced by females. Sex allocation hypotheses implicitly assume that each female shifts sex ratios from parity in response to sex allocation cues; my results show that some females may shift from already skewed sex ratios. Thus, subsequent shifts in litter sex ratios in response to sex allocation cues may also reflect the influence of prenatal hormone exposure on sex ratios, potentially producing some of the inconsistencies observed between theoretical and empirical mammalian sex allocation.

Introduction

Adaptive sex ratio biases are predicted if there are sex-specific fitness returns from investment into sons versus daughters (e.g. Charnov *et al.*, 1981; Clark, 1978; Hamilton, 1967; Trivers & Willard, 1973). For example, in polygynous species where male reproductive success is more variable and high maternal body condition enhances the lifetime fitness of sons more than daughters, mothers in good condition are predicted to have more sons and mothers in poor condition are predicted to have more daughters (Trivers & Willard, 1973). While there is generally strong concordance between predictions from sex allocation theory and empirical observations (reviewed in West, 2009), studies in mammals often produce empirical results that are inconsistent with theoretical predictions (e.g. Brown & Silk, 2002; Cameron, 2004; Hewison & Gaillard, 1999). While some of these inconsistent results are attributed to differences in methodology between studies (Cameron, 2004; Sheldon & West, 2004), the remaining unexplained inconsistencies may, in part, be due to physiological constraints on some females' capacity to adjust sex ratios to the extent theory predicts (Edwards *et al.*, 2016b).

Sex allocation predictions implicitly assume that all females are equally able to physiologically adjust sex ratios in respond to sex allocation cues (see West, 2009). Several physiological factors have been linked to sex ratios skews in mammals (reviewed in Cameron *et al.*, 2017; Navara, 2018), with observed skews frequently consistent with theoretical predictions (e.g. Cameron, 2004). For example, variation in maternal periconceptional blood glucose, which is influenced by nutrition and body condition, is linked to offspring sex ratios in that higher glucose is associated with more sons and lower glucose is associated with more daughters

(Cameron *et al.*, 2008; Helle *et al.*, 2008), consistent with the predictions of condition-dependent sex allocation (Cameron, 2004). Additionally, higher maternal testosterone is associated with a greater proportion of sons in a variety of mammalian species (Grant & Irwin, 2005; Helle *et al.*, 2008; Shargal *et al.*, 2008; for a meta-analysis see Merkling *et al.*, 2018), potentially providing a link between the predicted relationship between maternal social status and offspring sex ratios for some species (Grant, 2007; Sheldon & West, 2004). However, factors unrelated to sex allocation also influence the hypothesised physiological links between the local maternal environmental and offspring sex ratios, posing potential physiological constraints on maternal sex allocation (Edwards *et al.*, 2016b). For example, the a females *in utero* exposure to hormones can affect the sex ratios she produces as an adult (Clark & Galef, 1995; Dunn *et al.*, 2010; Edwards *et al.*, 2016a; Vandenbergh & Huggett, 1994) and may further affect her capacity to adjust sex ratios according to cues of sex allocation (Edwards *et al.*, 2019).

The sex hormones, testosterone and oestradiol, play an important role in sexual differentiation beyond the development of the ovary or testis (Arnold & McCarthy, 2016; MacLusky & Naftolin, 1981). Within each sex, differential exposure to prenatal testosterone and oestradiol influences developmental trajectories, resulting in lifelong effects on morphology, physiology, behaviour, and reproductive traits (reviewed in Beatty, 1979; Evans *et al.*, 2016; Ryan & Vandenbergh, 2002; Vandenbergh, 2003). In eutherian mammals, differential exposure to prenatal testosterone and oestradiol occurs from variation in endogenous, maternal, placental, and, in litter-bearing species, neighbouring siblings *in utero* (Hu *et al.*, 2015; Monclús & Blumstein, 2012; Vom Saal, 1983; Vreeburg *et al.*, 1983). Consequently, both males and females vary in the degree of phenotypic masculinisation and

feminisation (Ryan & Vandenbergh, 2002; Vandenbergh, 2003). In female rodents, the masculinising effects of exposure to a higher ratio of testosterone to oestradiol during late prenatal development include a lower ratio of the second to fourth digit (digit ratio; Huber *et al.*, 2017; Zheng & Cohn, 2011), irregular oestrous cycling (Witham *et al.*, 2012), higher concentrations of and sensitivity to testosterone (Gandelman *et al.*, 1979; Sullivan & Moenter, 2004; Witham *et al.*, 2012), elevated fasting glucose (Roland *et al.*, 2010), and a tendency to produce more sons (Clark & Galef, 1995; Vandenbergh & Huggett, 1994). Exposure to a lower oestradiol to testosterone ratio during late prenatal development has opposing feminising effects on female morphology and physiology (Clark & Galef, 1995; Huber *et al.*, 2017; Roland *et al.*, 2010; Sullivan & Moenter, 2004; Vandenbergh & Huggett, 1994; Witham *et al.*, 2012; Zheng & Cohn, 2011). Thus, the degree to which a female is masculinised or feminised may influence sex allocation if the capacity for sex ratio adjustment is affected.

There are also several differences between male and female non-reproductive behaviours that display similar masculinised and feminised effects due to prenatal testosterone and oestradiol exposure (reviewed in Arnold & Breedlove, 1985; Beatty, 1979; Celec *et al.*, 2015; Domonkos *et al.*, 2018). In rodents of both sexes, exposure to a higher ratio of testosterone to oestradiol starting in late prenatal development and continuing through neonate development is associated with higher anxiety and lower activity as adults and are considered masculinised behaviours (Beatty, 1979; Domonkos *et al.*, 2018; Lucion *et al.*, 1996; Zimmerberg & Farley, 1993; Zuloaga *et al.*, 2011). A higher ratio of oestradiol to testosterone during perinatal development has the opposing effects of lower anxiety and higher activity in adulthood and are considered feminised behaviours (Beatty, 1979; Domonkos *et al.*, 2018;

Lucion *et al.*, 1996; Zimmerberg & Farley, 1993; Zuloaga *et al.*, 2011). These behaviours are further influenced by the relative concentrations of testosterone and oestradiol in adulthood, thereby establishing the observed sex differences in rodent anxiety- and activity-type behaviours (Arnold & Breedlove, 1985; Beatty, 1979; Goel & Bale, 2008).

To assess whether variation in the degree of masculinisation or feminisation influences the offspring sex ratios produced in adulthood, I utilised the inherent differences in exposure to prenatal testosterone and oestradiol in mice with variation in intrauterine position, and maternal and placental sources, eliminating the need for an experimental treatment to address my aims. I collated data on litter sex ratios from experiments on female laboratory mice (*Mus musculus*) that showed natural variation in the degree of phenotypic masculinisation and feminisation. I used digit ratio as the primary measurement of the degree of masculinisation and feminisation; digit ratio is established concurrently to the organisational effects of prenatal testosterone and oestradiol exposure (e.g. Roland *et al.*, 2010; Sullivan & Moenter, 2004; Witham *et al.*, 2012) that might subsequently influence sex allocation (e.g. Cameron *et al.*, 2008; Helle *et al.*, 2008). I also recorded anxiety and activity behaviours to further inform the degree of masculinisation/feminisation (Beatty, 1979; Domonkos *et al.*, 2018; Fernandes *et al.*, 1999). I hypothesised that females with more masculinised digit ratios will display more masculinised behaviour, specifically elevated anxiety and reduced activity, and produce more sons, and females with more feminised digit ratios will display more feminised behaviour, specifically reduced anxiety and elevated activity, and produce more daughters.

Methods

Study system

I used data opportunistically sourced from BALB/c laboratory-bred mice collated from two studies conducted by myself and my research group. The first study was a failed pilot study on the effects of orally administered testosterone (0.5 mg; Keisler, Kier & Walker 1991; Livera et al. 2006; n=4) and melengestrol acetate (0.2ug; Patton et al. 2001; Perry et al. 2002; Pfaffl et al. 2002; n=4), which reduces maternal circulating testosterone and oestradiol (Matsuyama, Richkind & Cupps 1967; Knol & Egberink-Alink 1989; Pfaffl et al. 2002), as well as no experimental treatment (n=4) on prenatal days 16.5 to 18.5. These 12 females then produced 27 females who later produced the litters that comprised the litter sex ratio data used in this study. Analyses showed that treatment had no effect on digit ratios or litter sex ratios (see below), likely due to the fact that other factors that influence foetal exposure to testosterone and oestradiol were not controlled for, including but not limited to intrauterine position, placental testosterone synthesis, endogenous maternal hormones (Bridges and Russel 1981; Gibori and Sridaran, 1981; Houtsmiller *et al*., 1995; Stocco 2008; Vom Saal, 1983; Vreeburg *et al*., 1983). The data from these 27 females were combined with data from the litters produced by 15 control female mice from Edwards (2016). All mice were derived from a source population at the University of Tasmania's Animal Services Cambridge Farm Facility with breeding and handling protocols (see below) identical for the two studies. Thus, data from litters produced by 36 female mice were collated and I used the natural variation in prenatal masculinisation and feminisation, as measured by digit ratio, in this population to assess the relationship between prenatal testosterone and oestradiol exposure and litter sex ratios.

Mice were housed at the University of Tasmania Department of Biological Sciences in OptiMice® cages (14.5 cm x 31 cm x 16.5 cm) with a MicroVent IVC® system with one-way airflow and kept under a 12 hr L:D photoperiod at approximately 25 °C. Barastoc® irradiated feed and filtered water were provided ad libitum. Adult female mice were housed three to five per cage, males were housed three to four per cage. Males that were used for breeding were separated and housed individually after breeding. Breeding protocol is detailed below.

Digit ratio

The ratio of the second to fourth digit on the right hind foot of each female was measured with digit callipers after sexual maturity (day 35). Digit ratio provided the primary measure of the degree of masculinisation or feminisation of each female. In mice, digit ratio is a sex-specific trait established by the ratio of testosterone to oestradiol exposure during late prenatal development (Brown *et al.*, 2002; Huber *et al.*, 2017; Zheng & Cohn, 2011). BALB/c mice exhibit a sex difference in digit ratio consistent with lower digit ratios as masculinised and higher digit ratios as feminised (Brown *et al.*, 2002; Yan *et al.*, 2009). Prior to the commencement of statistical analyses on behaviour and litter sex ratios, I tested whether prenatal exposure to the testosterone or melengestrol acetate treatments in the first experiment altered female digit ratios. As it did not (ANOVA: $F_{(2, 33)} = 0.83$, $Pr(>F) = 0.23$), all females from the first experiment were included in all subsequent analyses.

Behaviour

The 27 females from the first experiment and all their offspring underwent standardised behavioural testing, as detailed below. No females from the second experiment were behaviourally tested. Behavioural scores of the 27 females plus the daughters of the control females (n =10; to increase statistical power) were used to provide an additional measure of masculinisation and feminisation. Each female was subject to testing in each of three standardized behavioural tests: open field, elevated plus maze, and holeboard (Carola *et al.*, 2002; Fernandes *et al.*, 1999; Sestakova *et al.*, 2013). These behavioural tests assess anxiety and activity by exploiting the contrasting tendency of mice to explore novel areas against the aversion to open, exposed, and elevated spaces; mice experiencing higher anxiety are less active as they are less inclined to explore novel and risky areas (e.g. open, elevated spaces; File, 2001; Lister, 1987). Six behaviours were selected *a priori* to represent related but distinct aspects of anxiety- and activity-type behaviours that are influenced by perinatal testosterone and oestradiol (Table 2.1).

Table 2.1. Summary of the specific aspects of anxiety- and activity- type behaviours recorded in each standardised test. Each behaviour was compared to the ratio of the second to fourth digit (digit ratio) of female laboratory mice to provide an additional measure of masculinisation and feminisation.

Open field test	
Time in centre ring	Proportion of time all four paws were within the centre ring divided by the number of entries into the centre. More time in the centre represents higher activity and non-directed exploration, and lower agoraphobia (Carola *et al.*, 2002; Prut & Belzung, 2003; Sestakova *et al.*, 2013).
Time on edge	Proportion of time the side of the body was in contact with the outer wall divided by entries to the edge squares. More time on the edge represents more hesitant activity relating to higher anxiety (Carola *et al.*, 2002; Sestakova *et al.*, 2013).
Time not moving	Proportion of time the mouse was not moving, excluding grooming time. More time not moving represents a higher fear response (Sestakova *et al.*, 2013).
Elevated plus maze	
Closed arm entries	Number of times all four feet enter the closed arm from the centre square. More entries represent higher activity (Cruz *et al.*, 1994; Fernandes & File, 1996; File, 2001).
Time in open arm	Proportion of time all feet exited the centre square and moved onto an open arm of maze divided by the number of times all four feet moved onto the open arm. More time in open arm represents lower anxiety associated with approach-avoidance conflict (Cruz *et al.*, 1994; Fernandes & File, 1996; File, 2001; Garcia *et al.*, 2005; Lister, 1987).
Holeboard	
Number of head dips	Number of times head dipped into a hole; new head dip was counted once head fully re-immerged from previous dip. More head dips represent lower neophobia and higher directed exploration (Durcan & Lister, 1989; File & Wardill, 1975; Takeda *et al.*, 1998; van der Staay *et al.*, 2012).

All females were behaviourally tested after sexual maturity (day 35) and before breeding. Testing was performed in an adjacent room to housing. Each mouse was brought into the testing room 20 minutes prior to testing to acclimate, and all testing took place between the hours of 9 am and 4 pm. All tests were video-recorded for later behavioural analysis. Between all trials, test apparatus was cleaned with F10-SC Veterinary Disinfectant ® (Chemical Essentials Pty Ltd, Melbourne, AUS). All behaviours were scored blindly (Holman *et al.*, 2015) from video recordings (HERO3 White ®).

Open field

Each mouse was placed in the centre of a round PVC open field arena, measuring 1 m in diameter and 20 cm high with 10 cm^2 squares delineating a locomotion unit and delineated centre circle with a diameter of 40 cm, and allowed to move freely for five minutes (see Figure 2.1). The behaviours recorded are listed in Table 2.1.

Elevated plus maze

A plus-shaped maze with two opposing open arms and two opposing closed arms, with each arm measuring 45 cm long x 16 cm high x 7 cm wide, was used (see Figure 2.1). Trials took place three days after the open field. Each mouse was placed in the centre of the plus and allowed to explore the maze for five minutes. Recorded behaviours are listed in Table 2.1.

Holeboard

Each mouse was placed at the centre of a PVC square measuring 40 cm long x 40 cm wide and consisting of 16 equidistance holes 3 cm in diameter and suspended 50 cm above the

floor. Each mouse was allowed to roam freely for five minutes (see Figure 2.1). Trials took place three days after the elevated plus maze. One behaviour was recorded (Table 2.1).

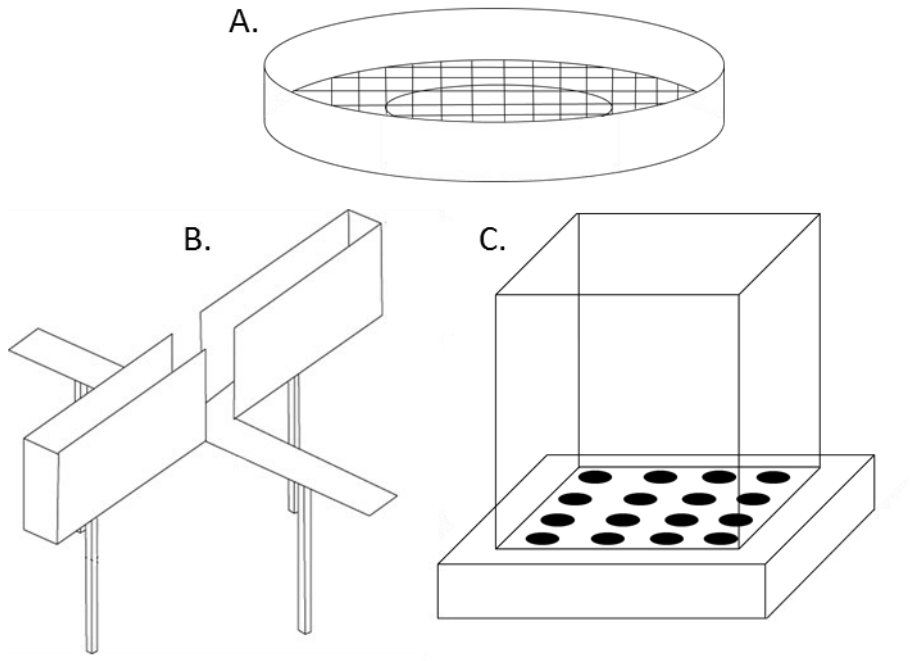

Figure 2.1. Visual depiction of the three standardised behavioural tests: A) open field test B) elevated plus maze and C) holeboard.

Litter sex ratios

The females in both experiments were bred to males who had not been exposed to any hormone treatment. All males and females were over the age of 35 days at the time of breeding. During breeding, females were housed in pairs with a male until the presence of a copulatory plug was observed. Females were then returned to home cages and weight monitored until pregnancy was apparent; pregnant females were then housed individually.

In total, 188 pups were born at full term to 36 females. No female produced more than one litter. Among the 188 pups that were born, 27 pups were either stillborn or died shortly after birth. All but three of the deceased pups were successfully sexed using PCR amplification using the X-specific Jardi1c and Y-specific Jardi1d primers (Clapcote & Roder, 2005) and were included in sex ratio analysis. This resulted in a total of 185 sexed pups from 36 females.

Ethical note

All procedures adhered to the Australian Code for the Care and Use of Animals for the Scientific Purposes and were approved by the University of Tasmania Ethics Committee under permit numbers A0014877 and A0012366.

Statistical Analysis

Digit ratio

All analyses were performed in R (R Core Team, 2017). Digit ratios of females in each treatment group were compared with an ANOVA using the *aov* function to determine any effect of treatment on digit ratios.

Behaviour

Behavioural scores (see Table 2.1) were transformed for normality where required: the proportion of time in the centre and edge of the open field were inverse square root transformed, and time spent not moving was log-transformed to normality. To test the relationship between digit ratio and behavioural scores, linear (LMMs) and generalized linear

mixed models (GLMMs) were fit for each behaviour using the *lme4* package (Bates *et al.*, 2014). Poisson GLMMs for count data were specified for the number of head dips and the number of entries into the closed arm. Due to the zero-inflated nature of the data for the proportion of time spent on the open arm, a two-part gamma hurdle mixed model for zero-inflated continuous data was specified, consisting of separate binomial and gamma GLMMs (McDowell, 2003; Mullahy, 1986). All other behaviours were normally distributed and modelled using LMMs. For each behaviour, four candidate models, including the null model, were constructed (see Table 2.2). Female identity was included as a random effect in all models. Models were ranked according to Akaike's information criterion corrected for small sample sizes (AIC_c) and their differences (Δ_i; Burnham & Anderson, 2002). Models were considered competitive if Δ_i was < 2.0 (Burnham & Anderson, 2002). When the difference in AIC_c (Δ_i) between models was less than 2, the model with the lowest number of parameters was retained as the most competitive model (parsimony criterion; Burnham & Anderson, 1998). Relative support for each model derived from the global model was assessed using weights derived from AIC_c scores (w_i; Burnham & Anderson, 2002). The relative effect of each predictor in the global model was quantified by summing the w_i values using the *importance* function in the *MuMin* library (Barton 2018).

Litter sex ratios

Four candidate GLMMs, including the null model, were specified with binomial error (see Table 2.3a) to examine the relationship between digit ratio (log-transformed) and litter sex ratio. The *cbind* function in the *lme4* package (Bates *et al.*, 2014) was used so that the response variable of litter sex ratio also contained information about the number of sons and

daughters leading to the ratio in each litter. Female body condition prior to conception (weight divided by right hind foot length, scaled by standard deviation and centred around zero) was included as a predictor of sex ratios (see Cameron, 2004); although all females have equal access to food in a laboratory setting, there is natural variation between the body conditions of females that can influence litter sex ratios (see Edwards *et al*., 2019). Treatment was included to account for the different treatments each female was exposed to *in utero,* even though previous analyses demonstrated treatment did not affect digit ratio (see above). The litter produced by each female was the unit of analysis and each female's own litter identity was included as a random effect to account for the relatedness among some females. Model selection followed as described above. The relationship between litter size and litter sex ratio was assessed with a Poisson GLMM. The relationship between digit ratio and litter size was assessed with a binomial GLMM.

Twenty-one of the 36 females that were included in the litter sex ratio analysis were also behaviourally tested. To assess whether the one behavioural trait that showed masculinised and feminised effects was further linked to litter sex ratios, a GLMM was fit with the number of head dips as a fixed factor and female identity as a random effect predicting the litter sex ratios of the subset of the 21 females. This model was compared as described above to a GLMM fit with digit ratio as the fixed factor predicting the litter sex ratios of the same 21 females.

Results

Digit ratio

Digit ratios among all females ranged from 0.795 to 1.3. The range of digit ratios of females that had been exposed to either testosterone or MGA *in utero* did not vary from those of the controls: treatment digit ratio = 0.87 - 1.36; control digit ratio = 0.9 - 1.31. The mean digit ratio among the testosterone exposed (1.07 ± 0.09), MGA exposed (1.04 ± 0.15), and controls (0.99 ± 0.13) also did not differ (ANOVA: $F_{(2, 33)} = 0.83$, Pr(>F) = 0.23). The digit ratios from each of the two experiments also did not differ (ANOVA: $F_{(1, 34)} = 0.51$, Pr(>F) = 0.48).

Behaviour

Females with more feminised digit ratios explored more in the holeboard, as evident by the higher number of head dips (Figure 2.2). Digit ratio had a relative importance of 99 % and the model containing only this parameter carried 91 % of the weight among the model set (Table 2.2). No other recorded behaviours were linked to digit ratio, as evidenced by the null model being the highest ranked model for all other behaviours (Table 2.2).

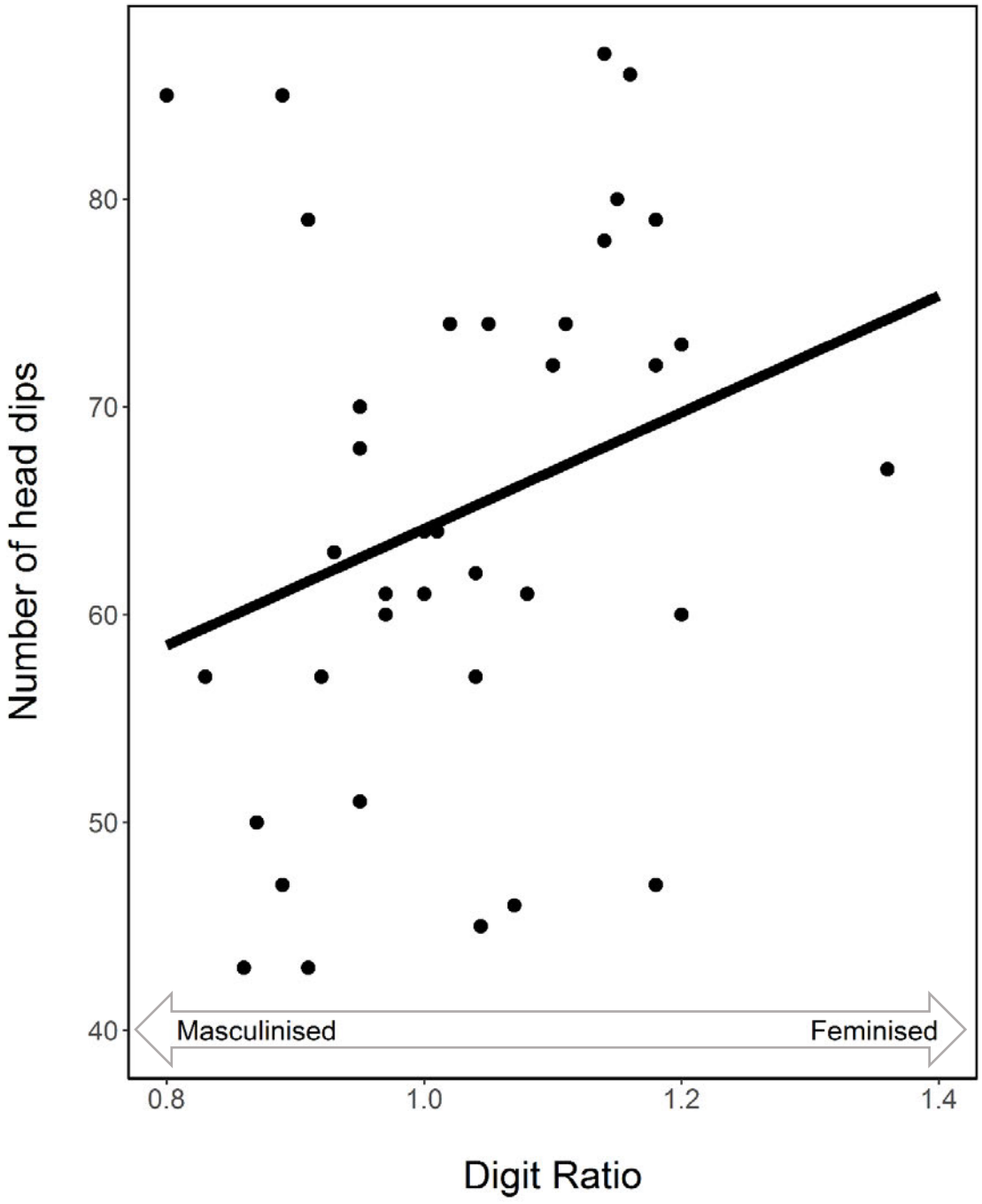

Figure 2.2. The linear relationship between the ratio of the second to fourth digit of the right hind foot (digit ratio) and the number of head dips in the holeboard test among 37 female BALB/c mice.

Table 2.2. Results of model selection for each of the behaviours recorded in the standardised tests to determine whether digit ratio was linked to aspects of anxiety- and activity- type behaviours.

Test	Behaviour	Model Type	Model	df	log likelihood	AIC_c	Δ_i	w_i
Holeboard	Number of head dips	Poisson GLMM	~ digit ratio	3	-146.7	300.2	0.00	0.91
			~ digit ratio + treatment	5	-146.7	305.2	5.04	0.07
			~1	2	-152.3	308.9	8.81	0.01
Open field	Time not moving	LMM	~1	3	1.3	4.1	0.00	0.76
			~ digit ratio	4	1.4	6.4	2.30	0.24
			~ digit ratio + treatment	6	-0.8	16. 4	12.35	0.00
	Time in centre ring	LMM	~1	3	17.0	-27.3	0.00	0.88
			~ digit ratio	4	16.3	-23.4	3.91	0.12
			~ digit ratio + treatment	6	12.6	-10.4	16.92	0.00
	Time on edge	LMM	~1	3	19.2	-31.7	0.00	0.87
			~ digit ratio	4	18.6	-27.9	3.73	0.13
			~ digit ratio + treatment	6	15.5	-16.2	15.46	0.00
Elevated plus maze	Closed arm entries	Poisson GLMM	~1	2	-113.0	230.4	0.00	0.35
			~ digit ratio + treatment	5	-109.3	230.5	0.08	0.33
			~ digit ratio	3	-112.6	231.8	1.45	0.17
	Open arm entries	Gamma hurdle (Binomial)	~1	2	-24.5	53.4	0.00	0.74
			~ digit ratio	3	-24.5	55.8	2.32	0.23
			~ digit ratio + treatment	5	-24.4	60.8	7.35	0.02
		(Gamma)	~1	3	-56.6	120.5	0.00	0.72
			~ digit ratio	4	-56.1	122.5	2.01	0.26
			~ digit ratio + treatment	6	-55.3	127.8	7.27	0.02

Litter sex ratios

All litter sex ratios are reported as the proportion of daughters per litter. In total, 185 pups from 36 females were included in litter sex ratio analyses. Litter size ranged from one to ten pups. Litter size was not associated with digit ratio (GLMM: $F_{(1, 34)} = 1.14$, Pr(>F) = 0.28), nor with litter sex ratios (GLMM: $F_{(1, 34)} = 0.31$, Pr(>F) = 0.31). Litter sex ratios ranged from 0 to 1. Results from model selection suggested that digit ratio best predicted litter sex ratios (Table 2.3a). The model containing this single predictor ranked the highest, with 54 % of the weight among the model set and a relative importance of 78 %, compared to 20 % for maternal condition and 10 % for treatment. All other candidate models, including the null model, fell outside of the Δ_i threshold of 2.0 and were not competitive models. Digit ratio predicted the proportion of daughters in a litter: females with more feminised digit ratios produced more daughters (OR: 0.07; 95 % CI: 0.01 - 0.9; P = 0.03; Figure 2.3); digit ratio explained 3 % of the variation in litter sex ratios (GLMM: $r^2 = 0.03$). Treatment exposure *in utero* did not affect litter sex ratios (Table 2.3a). The number of head dips, although related to digit ratio (see Table 2.2), was not correlated to a female's litter sex ratio (Table 2.3b.)

Table 2.3. Results of model selection to determine a) which set of selected factors influenced the litter sex ratios produced by 36 BALB/c female mice and b) compare the relationship between the number of heads and litter sex ratio, and digit ratio and litter sex ratio among the 21 females that both produced litters and were behaviourally tested.

	Model	df	log likelihood	AIC_c	Δ_i	w_i
a)	~ digit ratio	3	-47.6	102.0	0.00	0.54
litter sex ratios	~ 1	2	-49.9	104.1	2.15	0.17
	~ digit ratio + condition	4	-47.5	104.2	2.29	0.19
	~ digit ratio + treatment	5	-49.7	106.2	4.22	0.07
	~ condition + treatment	5	-47.8	107.7	5.71	0.02
b)	~ digit ratio	3	-25.3	58	0	0.92
litter sex ratios	~ head dips	3	-27.8	63	4.9	0.08

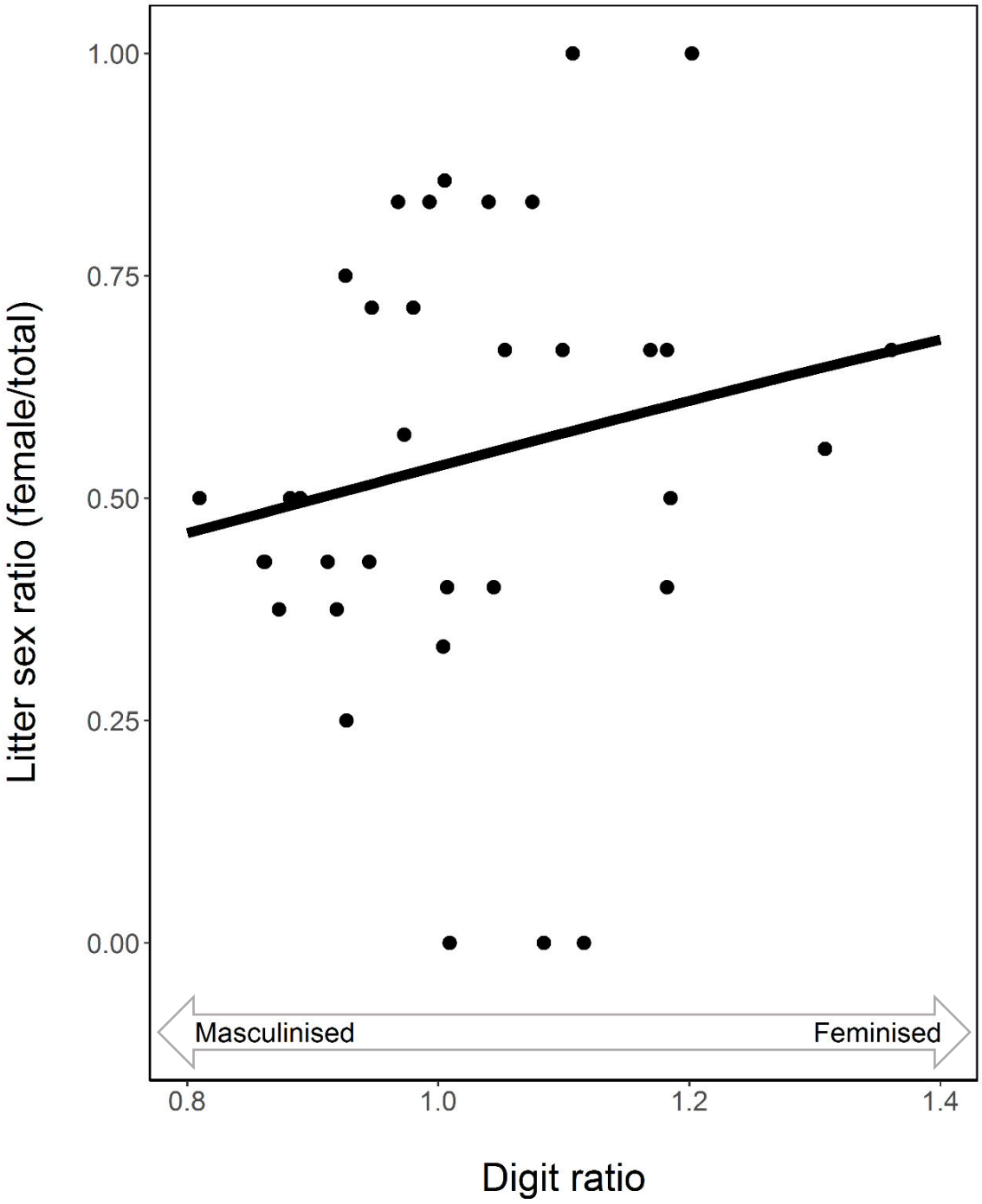

Figure 2.3. The ratio of the second to fourth digit of the right hind foot (digit ratio) plotted against the proportion of daughters in the litters of 36 female BALB/c mice.

Discussion

Litter sex ratios

I tested whether digit ratio was associated with litter sex ratio in female mice. I found that females with more feminised digit ratios produced more daughters. Digit ratio is representative of the masculinising and feminising effects of late prenatal testosterone and oestradiol exposure (Brown *et al.*, 2002; Huber *et al.*, 2017; Zheng & Cohn, 2011), which, in mice, varies from differential exposure from endogenous, maternal, placental, and neighbouring siblings in utero (Hu et al., 2015; Monclús & Blumstein, 2012; Vom Saal, 1983; Vreeburg et al., 1983). Thus, these results suggest that variation in prenatal exposure to testosterone and oestradiol influences future litter sex ratios, a result consistent with similar studies in rodents and humans (Clark & Galef, 1995; Kim *et al.*, 2015; Lutchmaya *et al.*, 2004; Manning *et al.*, 2002; Vandenbergh & Huggett, 1994; Ventura *et al.*, 2013). Specifically, my results link a greater exposure to oestradiol relative to testosterone during late prenatal developmental to more daughters in adulthood. This result suggests that some variation in offspring sex ratios is related to a female's prenatal hormone exposure, potentially influencing the female's ability to respond facultatively to proximate modifiers of sex allocation. While sex allocation hypotheses implicitly assume that each female shifts offspring sex ratios from parity, my results indicate that some females may shift from already skewed sex ratios, thereby introducing complications to sex allocation predictions and potentially producing empirical results that are inconsistent with theoretical predictions.

The nature of the specific organisational effects of prenatal testosterone and oestradiol exposure on reproductive physiology that mechanistically explain the relationship between

digit ratios and litter sex ratios was beyond the scope of this experiment. However, other studies in mice demonstrate that variation in testosterone and oestradiol exposure during late prenatal development affects adult testosterone and glucose concentrations (Cederroth & Nef, 2009; Moore *et al.*, 2013; Roland *et al.*, 2010; Sullivan & Moenter, 2004; Witham *et al.*, 2012), both of which are implicated in rodent sex allocation in (Cameron *et al.*, 2008; Helle *et al.*, 2008). For example, prenatally masculinised female mice show increased concentrations of and sensitivity to testosterone (Moore *et al.*, 2013; Sullivan & Moenter, 2004; Witham *et al.*, 2012), increased fasting glucose, and reduced glucose tolerance in adulthood (Roland *et al.*, 2010), whereas prenatally feminised mice show increased glucose tolerance in adulthood (Cederroth & Nef, 2009). As reduced maternal testosterone and glucose are linked to a greater proportion of daughters (Cameron *et al.*, 2008; Grant *et al.*, 2008; Helle *et al.*, 2008; Firman, 2020), subtle differences between females in adult testosterone or glucose concentrations relating to prenatal masculinisation/feminisation could have contributed to the variation in litter sex ratios recorded in the present study. Further work is required to assess the nature of the organisational effects of prenatal testosterone and oestradiol exposure on reproductive physiology that facilitate sex ratio skews in adulthood.

Behaviour

Six different behaviours relating to prenatal testosterone and oestradiol exposure were recorded to further inform the degree of masculinisation/feminisation of each female. These six behaviours were recorded as each represents related but distinct aspects of anxiety- and activity-type behaviours (see Table 2.1). Of the recorded behaviours, only the number of

head dips in the holeboard test was linked to digit ratio; females with more feminised digit ratio displayed more head dips. As more head dips represent reduced neophobia and a greater tendency for directed exploration, considered elsewhere to be feminised rodent behaviour (Fernandes *et al.*, 1999; Takeda *et al.*, 1998), this result provides some support to my hypothesis. However, the number of head dips did not predict litter sex ratios, suggesting that the number of head dips in the holeboard test provides a relatively poor indicator of the masculinising and feminising prenatal effects that influence sex ratios.

There were no links between digit ratio and any of the behaviours representing other aspects of anxiety- and activity-type behaviours. There are at least two explanations for these results. Firstly, each of the distinct behaviours recorded by the open field, elevated plus maze, and holeboard show variability in the extent to which they are influenced by testosterone and oestradiol exposure during pre- versus postnatal development (e.g. Domonkos *et al.*, 2018; Farabollini *et al.*, 1999; Xu *et al.*, 2012). For example, in mice, exposure to the estrogenic chemical bisphenol A affects behaviours in the open field and elevated plus maze when exposure occurs during both pre- and postnatal development (Gioiosa *et al.*, 2007; Nakamura *et al.*, 2012; Ryan & Vandenbergh, 2006; Tian *et al.*, 2010), but these behaviours are not affected if exposure occurs only during prenatal development (Cox *et al.*, 2010; Wolstenholme *et al.*, 2011). Thus, the behaviours in the open field and elevated plus maze may not be influenced by testosterone and oestradiol specifically during late prenatal development, as suggested by my results. Secondly, hormone fluctuations in adulthood, such as those during oestrous cycling, also differentially affect aspects of anxiety- and activity-type behaviours (Domonkos *et al.*, 2018; Frye *et al.*, 2000; Meziane *et al.*, 2007). For example, in rats, the stage of oestrous affects the behaviours in the open field and elevated plus maze,

but not head dips in the holeboard (Frye *et al.*, 2000). Female mice in this study were tested at or soon after sexual maturity and thus there was likely some variation in the stage of oestrous among females. Therefore, unaccounted for variation in oestrous cycling could have obscured the relationship between digit ratio and the behaviours in the open field and elevated plus maze. Overall, my results suggest that the behavioural measures of masculinisation and feminisation recorded here are not strong indicators of the masculinising and feminising prenatal effects that influence sex ratios in adulthood.

Conclusions

I showed that a female's digit ratio correlated with the litter sex ratio she produced, suggesting that prenatal testosterone and oestradiol exposure has implications for sex allocation. Firstly, some of the observed variation in offspring sex ratios may be explained by a female's prenatal hormone experience rather than current conditions predicting sex allocation. Secondly, some females may be physiologically constrained in their capacity to adjust sex ratios in response to sex allocation cues if, for example, females with feminised digit ratios are less able to produce more sons. Future work should clarify the extent to which females are constrained by exposing females with both masculinised and feminised digit ratios to conditions that predict more sons, such as higher caloric diets or diets high in fatty acids (Rosenfeld & Roberts, 2004), to test whether females with feminised digit ratios are less able to shift sex ratios towards sons. If prenatal hormone exposure affects the scope of some females' capacity to adjust sex ratios according to current conditions predicting sex allocation, as is suggested my work and the recent work of others (Edwards *et al.*, 2016a; Edwards *et al.*, 2019), variation among females in their prenatal hormone exposure might

explain some of the remaining inconsistencies between empirical results and theoretical predictions in mammalian sex allocation.

References

Arnold, A. P., & Breedlove, S. M. (1985). Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. *Hormones and Behavior, 19*, 469-498.

Arnold, A. P., & McCarthy, M. M. (2016). Sexual differentiation of the brain and behavior: a primer. In P. D. & V. N. (Eds.), *Neuroscience in the 21st Century* (pp. 2139-2168). New York, USA: Springer.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*, 1-48.

Beatty, W. W. (1979). Gonadal hormones and sex differences in nonreproductive behaviors in rodents: organizational and activational influences. *Hormones and Behavior, 12*, 112-163.

Bridges, R. S., & Russell, D. W. (1981). Steroidal interactions in the regulation of maternal behaviour in virgin female rats: effects of testosterone, dihydrotestosterone, oestradiol, progesterone and the aromatase inhibitor, 1, 4, 6-androstatriene-3, 17-dione. *Journal of Endocrinology*, 90, 31-40.

Brown, G. R., & Silk, J. B. (2002). Reconsidering the null hypothesis: is maternal rank associated with birth sex ratios in primate groups? *Proceedings of the National Academy of Sciences, 99*, 11252-11255.

Brown, W. M., Finn, C. J., & Breedlove, S. M. (2002). Sexual dimorphism in digit-length ratios of laboratory mice. *The Anatomical Record: An Official Publication of the American Association of Anatomists, 267*, 231-234.

Burnham, K. P., & Anderson, D. R. (1998). Practical use of the information-theoretic approach. In *Model Selection and Inference* (pp. 75-117). New York, USA: Springer.

Burnham, K. P., & Anderson, D. R. (2002). *Model Selection and Multimodel Inference* (2 ed.). New York, USA: Springer.

Cameron, E. Z. (2004). Facultative adjustment of mammalian sex ratios in support of the Trivers–Willard hypothesis: evidence for a mechanism. *Proceedings of the Royal Society B: Biological Sciences, 271*, 1723-1728.

Cameron, E. Z., Edwards, A. M., & Parsley, L. M. (2017). Developmental sexual dimorphism and the evolution of mechanisms for adjustment of sex ratios in mammals. *Annals of the New York Academy of Sciences, 1389*, 147-163.

Cameron, E. Z., Lemons, P. R., Bateman, P. W., & Bennett, N. C. (2008). Experimental alteration of litter sex ratios in a mammal. *Proceedings of the Royal Society B: Biological Sciences, 275*, 323-327.

Cameron, E. Z., & Linklater, W. L. (2000). Individual mares bias investment in sons and daughters in relation to their condition. *Animal Behaviour, 60*, 359-367.

Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., & Renzi, P. (2002). Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behavioural Brain Research, 134*, 49-57.

Cederroth, C. R., & Nef, S. (2009). Fetal programming of adult glucose homeostasis in mice. *PLoS One*, 4, e7281.

Celec, P., Ostatníková, D., & Hodosy, J. (2015). On the effects of testosterone on brain behavioral functions. *Frontiers in Neuroscience, 9*, 1-17.

Charnov, E. L., Los-den Hartogh, R., Jones, W., & van den Assem, J. (1981). Sex ratio evolution in a variable environment. *Nature, 289*, 27-32.

Clapcote, S. J., & Roder, J. C. (2005). Simplex PCR assay for sex determination in mice. *Biotechniques, 38*, 702-706.

Clark, A. B. (1978). Sex ratio and local resource competition in a prosimian primate. *Science, 201*, 163-165.

Clark, M. M., & Galef, B. G. (1995). A gerbil dam's fetal intrauterine position affects the sex ratios of litters she gestates. *Physiology & Behavior, 57*, 297-299.

Cox, K. H., Gatewood, J. D., Howeth, C., & Rissman, E. F. (2010). Gestational exposure to bisphenol A and cross-fostering affect behaviors in juvenile mice. *Hormones and Behavior, 58*, 754-761.

Cruz, A. P., Frei, F., & Graeff, F. G. (1994). Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacology Biochemistry and Behavior, 49*, 171-176.

Domonkos, E., Hodosy, J., Ostatníková, D., & Celec, P. (2018). On the role of testosterone in anxiety-like behavior across life in experimental rodents. *Frontiers in Endocrinology, 9*, 1-10.

Dunn, E., Kapoor, A., Leen, J., & Matthews, S. G. (2010). Prenatal synthetic glucocorticoid exposure alters hypothalamic–pituitary–adrenal regulation and pregnancy outcomes in mature female guinea pigs. *Journal of Physiology, 588*, 887-899.

Durcan, M. J., & Lister, R. G. (1989). Does directed exploration influence locomotor activity in a holeboard test? *Behavioral and Neural Biology, 51*, 121-125.

Edwards, A. M. (2016). *Constraints on maternal ability to adjust sex ratios in mammals* (Doctoral dissertation, University of Tasmania).

Edwards, A. M., Cameron, E. Z., Pereira, J. C., Wapstra, E., Ferguson-Smith, M. A., Horton, S. R., & Thomasson, K. (2016a). Gestational experience alters sex allocation in the subsequent generation. *Royal Society Open Science, 3*, 160210.

Edwards, A. M., Cameron, E. Z., & Wapstra, E. (2016b). Are there physiological constraints on maternal ability to adjust sex ratios in mammals? *Journal of Zoology, 299*, 1-9.

Edwards, A. M., Cameron, E. Z., Wapstra, E., & McEvoy, J. (2019). Maternal effects obscure condition-dependent sex allocation in changing environments. *Royal Society Open Science, 6*, 181885.

Evans, N. P., Bellingham, M., & Robinson, J. E. (2016). Prenatal programming of neuroendocrine reproductive function. *Theriogenology, 86*, 340-348.

Farabollini, F., Porrini, S., & Dessì-Fulgherit, F. (1999). Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats. *Pharmacology Biochemistry and Behavior, 64*, 687-694.

Fernandes, C., & File, S. E. (1996). The influence of open arm ledges and maze experience in the elevated plus-maze. *Pharmacology Biochemistry and Behavior, 54*, 31-40.

Fernandes, C., Gonzalez, M., Wilson, C., & File, S. E. (1999). Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. *Pharmacology Biochemistry and Behavior, 64*, 731-736.

File, S. E. (2001). Factors controlling measures of anxiety and responses to novelty in the mouse. *Behavioural Brain Research, 125*, 151-157.

File, S. E., & Wardill, A. G. (1975). The reliability of the hole-board apparatus. *Psychopharmacologia, 44*, 47-51.

Frye, C. A., Petralia, S. M., & Rhodes, M. E. (2000). Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3α, 5α-THP. *Pharmacology Biochemistry and Behavior, 67*, 587-596.

Gandelman, R., Simon, N. G., & McDermott, N. J. (1979). Prenatal exposure to testosterone and its precursors influences morphology and later behavioral responsiveness to testosterone of female mice. *Physiology & Behavior, 23*, 23-26.

Garcia, A. M. B., Martinez, R., Brandão, M. L., & Morato, S. (2005). Effects of apomorphine on rat behavior in the elevated plus-maze. *Physiology & Behavior, 85*, 440-447.

Gibori G and Sridaran R. (1981). Sites of androgen and estradiol production in the second half of pregnancy in the rat. *Biology of Reproduction, 24*, 249-256.

Gioiosa, L., Fissore, E., Ghirardelli, G., Parmigiani, S., & Palanza, P. (2007). Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. *Hormones and Behavior, 52*, 307-316.

Goel, N., & Bale, T. L. (2008). Organizational and activational effects of testosterone on masculinization of female physiological and behavioral stress responses. *Endocrinology, 149*, 6399-6405.

Grant, V. J. (2007). Could maternal testosterone levels govern mammalian sex ratio deviations? *Journal of Theoretical Biology, 246*, 708-719.

Grant, V. J., & Irwin, R. (2005). Follicular fluid steroid levels and subsequent sex of bovine embryos. *Journal of Experimental Zoology Part A: Comparative Experimental Biology, 303*, 1120-1125.

Grant, V. J., Irwin, R., Standley, N., Shelling, A., & Chamley, L. (2008). Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. *Biology of Reproduction, 78*, 812-815.

Hamilton, W. D. (1967). Extraordinary sex ratios. *Science, 156*, 477-488.

Helle, S., Laaksonen, T., Adamsson, A., Paranko, J., & Huitu, O. (2008). Female field voles with high testosterone and glucose levels produce male-biased litters. *Animal Behaviour, 75*, 1031-1039.

Hewison, A. M., & Gaillard, J. M. (1999). Successful sons or advantaged daughters? The Trivers-Willard model and sex-biased maternal investment in ungulates. *Trends in Ecology & Evolution, 14*, 229-234.

Holman, L., Head, M. L., Lanfear, R., & Jennions, M. D. (2015). Evidence of experimental bias in the life sciences: why we need blind data recording. *PLoS Biology, 13*, e1002190.

Houtsmuller, E. J., de Jong, F. H., Rowland, D. L., Slob, A. K. (1995). Plasma Testosterone in fetal rats and their mothers on day 19 of gestation. *Physiology and Behavior, 57*, 495-499.

Hu, M., Richard, J. E., Maliqueo, M., Kokosar, M., Fornes, R., Benrick, A., Jansson, T., Ohlsson, C., Wu, X., & Skibicka, K. P. (2015). Maternal testosterone exposure increases anxiety-like behavior and impacts the limbic system in the offspring. *Proceedings of the National Academy of Sciences, 112*, 14348-14353.

Huber, S. E., Lenz, B., Kornhuber, J., & Müller, C. P. (2017). Prenatal androgen-receptor activity has organizational morphological effects in mice. *PLoS One, 12, e0188752*.

Kim, T. B., Oh, J. K., Kim, K. T., Yoon, S. J., & Kim, S. W. (2015). Does the mother or father determine the offspring sex ratio? Investigating the relationship between maternal digit ratio and offspring sex ratio. *PLoS One, 10*, e0143054.

Lister, R. G. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology, 92*, 180-185.

Lucion, A. B., Charchat, H., Pereira, G. A., & Rasia-Filho, A. A. (1996). Influence of early postnatal gonadal hormones on anxiety in adult male rats. *Physiology & Behavior, 60*, 1419-1423.

Lutchmaya, S., Baron-Cohen, S., Raggatt, P., Knickmeyer, R., & Manning, J. T. (2004). 2nd to 4th digit ratios, fetal testosterone and estradiol. *Early Human Development, 77*, 23-28.

MacLusky, N. J., & Naftolin, F. (1981). Sexual differentiation of the central nervous system. *Science, 211*, 1294-1303.

Manning, J. T., Martin, S., Trivers, R. L., & Soler, M. (2002). 2nd to 4th digit ratio and offspring sex ratio. *Journal of Theoretical Biology, 217*, 93-95.

McDowell, A. (2003). From the help desk: hurdle models. *The Stata Journal, 3*, 178-184.

Merkling, T., Nakagawa, S., Lagisz, M., & Schwanz, L. E. (2018). Maternal testosterone and offspring sex-ratio in birds and mammals: a meta-analysis. *Evolutionary Biology, 45*, 96-104.

Meziane, H., Ouagazzal, A. M., Aubert, L., Wietrzych, M., & Krezel, W. (2007). Estrous cycle effects on behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping strategies. *Genes, Brain and Behavior, 6*, 192-200.

Monclús, R., & Blumstein, D. T. (2012). Litter sex composition affects life-history traits in yellow-bellied marmots. *Journal of Animal Ecology, 81*, 80-86.

Moore, A. M., Prescott, M., & Campbell, R. E. (2013). Estradiol negative and positive feedback in a prenatal androgen-induced mouse model of polycystic ovarian syndrome. *Endocrinology, 154*, 796-806.

Mullahy, J. (1986). Specification and testing of some modified count data models. *Journal of Econometrics, 33*, 341-365.

Nakamura, K., Itoh, K., Dai, H., Han, L., Wang, X., Kato, S., Sugimoto, T., & Fushiki, S. (2012). Prenatal and lactational exposure to low-doses of bisphenol A alters adult mice behavior. *Brain and Development, 34*, 57-63.

Navara, K. J. (2018). *Choosing Sexes: Mechanisms and Adaptive Patterns of Sex Allocation in Vertebrates*. New York, USA: Springer.

Prut, L., & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *European Journal of Pharmacology, 463*, 3-33.

R Core Team (2017). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*. Vienna, Austria.

Roland, A. V., Nunemaker, C. S., Keller, S. R., & Moenter, S. M. (2010). Prenatal androgen exposure programs metabolic dysfunction in female mice. *Journal of Endocrinology, 207*, 213-223.

Rosenfeld, C. S., & Roberts, R. M. (2004). Maternal diet and other factors affecting offspring sex ratio: a review. *Biology of Reproduction, 71*, 1063-1070.

Ryan, B. C., & Vandenbergh, J. G. (2002). Intrauterine position effects. *Neuroscience & Biobehavioral Reviews, 26*, 665-678.

Ryan, B. C., & Vandenbergh, J. G. (2006). Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. *Hormones and Behavior, 50*, 85-93.

Sestakova, N., Puzserova, A., Kluknavsky, M., & Bernatova, I. (2013). Determination of motor activity and anxiety-related behaviour in rodents: methodological aspects and role of nitric oxide. *Interdisciplinary Toxicology, 6*, 126-135.

Shargal, D., Shore, L., Roteri, N., Terkel, A., Zorovsky, Y., Shemesh, M., & Steinberger, Y. (2008). Fecal testosterone is elevated in high ranking female ibexes (*Capra nubiana*) and associated with increased aggression and a preponderance of male offspring. *Theriogenology, 69*, 673-680.

Sheldon, B. C., & West, S. A. (2004). Maternal dominance, maternal condition, and offspring sex ratio in ungulate mammals. *The American Naturalist, 163*, 40-54.

Stocco, C. (2008). Aromatase expression in the ovary: hormonal and molecular regulation. Steroids, 73, 473-487.

Sullivan, S. D., & Moenter, S. M. (2004). Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. *Proceedings of the National Academy of Sciences, 101*, 7129-7134.

Takeda, H., Tsuji, M., & Matsumiya, T. (1998). Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. *European Journal of Pharmacology, 350*, 21-29.

Tian, Y. H., Baek, J. H., Lee, S. Y., & Jang, C. G. (2010). Prenatal and postnatal exposure to bisphenol a induces anxiolytic behaviors and cognitive deficits in mice. *Synapse, 64*, 432-439.

Trivers, R. L., & Willard, D. E. (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science, 179*, 90-92.

van der Staay, F. J., Gieling, E. T., Pinzón, N. E., Nordquist, R. E., & Ohl, F. (2012). The appetitively motivated "cognitive" holeboard: a family of complex spatial discrimination tasks for assessing learning and memory. *Neuroscience & Biobehavioral Reviews, 36*, 379-403.

Vandenbergh, J. G. (2003). Prenatal hormone exposure and sexual variation: hormone exposure in the womb is instrumental in shaping the sexual anatomy, physiology and behavior of mammals. *American Scientist, 91*, 218-225.

Vandenbergh, J. G., & Huggett, C. L. (1994). Mother's prior intrauterine position affects the sex ratio of her offspring in house mice. *Proceedings of the National Academy of Sciences, 91*, 11055-11059.

Ventura, T., Gomes, M., Pita, A., Neto, M., & Taylor, A. (2013). Digit ratio (2D:4D) in newborns: influences of prenatal testosterone and maternal environment. *Early Human Development, 89*, 107-112.

Vom Saal, F. S. (1983). The interaction of circulating oestrogens and androgens in regulating mammalian sexual differentiation. In B. J., P. E., & G. R. (Eds.), *Hormones and Behaviour in Higher Vertebrates* (pp. 159-177). Berlin, Germany: Springer-Verlag.

Vreeburg, J., Groeneveld, J., Post, P., & Ooms, M. (1983). Concentrations of testosterone and androsterone in peripheral and umbilical venous plasma of fetal rats. *Journal of Reproduction and Fertility, 68*, 171-175.

West, S. (2009). *Sex Allocation*. Princeton, USA: Princeton University Press.

Witham, E. A., Meadows, J. D., Shojaei, S., Kauffman, A. S., & Mellon, P. L. (2012). Prenatal exposure to low levels of androgen accelerates female puberty onset and reproductive senescence in mice. *Endocrinology, 153*, 4522-4532.

Wolstenholme, J. T., Taylor, J. A., Shetty, S. R., Edwards, M., Connelly, J. J., & Rissman, E. F. (2011). Gestational exposure to low dose bisphenol A alters social behavior in juvenile mice. *PLoS One, 6*, e25448.

Xu, X., Hong, X., Xie, L., Li, T., Yang, Y., Zhang, Q., Zhang, G., & Liu, X. (2012). Gestational and lactational exposure to bisphenol-A affects anxiety-and depression-like behaviors in mice. *Hormones and Behavior, 62*, 480-490.

Yan, R. H., Bunning, M., Wahlsten, D., & Hurd, P. L. (2009). Digit ratio (2D:4D) differences between 20 strains of inbred mice. *PLoS One, 4*, e5801.

Zheng, Z., & Cohn, M. J. (2011). Developmental basis of sexually dimorphic digit ratios. *Proceedings of the National Academy of Sciences, 108*, 16289-16294.

Zimmerberg, B., & Farley, M. J. (1993). Sex differences in anxiety behavior in rats: role of gonadal hormones. *Physiology & Behavior, 54*, 1119-1124.

Zuloaga, D. G., Jordan, C. L., & Breedlove, S. M. (2011). The organizational role of testicular hormones and the androgen receptor in anxiety-related behaviors and sensorimotor gating in rats. *Endocrinology, 152*, 1572-1581.

Chapter 3: Ejaculate level variation in sperm sex ratios in domestic horses

Abstract

Sex allocation research has predominantly focused on how maternal fitness is enhanced by adjusting offspring sex ratios according to the relative fitness returns from investment into sons versus daughters. As the heterogametic sex, mammalian fathers might influence sex allocation through biased sperm sex ratios, which show variation in response to a range of factors, including age and mating frequency. Thus, there may be individual and ejaculate level variation in sperm sex ratios. I assessed sperm sex ratios in successive ejaculates collected from domestic stallions (*Equus ferus caballus*) of two populations that differed in location, breed, and ejaculation frequency. I increased the ejaculation frequency of all stallions over a three-day period to induce sexual exhaustion and measured the ratio of ejaculated X- to Y-chromosome-bearing-spermatozoa (CBS) in each successive ejaculate. The proportion of ejaculated X-CBS increased across successive ejaculates in the first population of stallions, while the second population showed a consistent X-CBS bias in their ejaculates. The difference in results between the two populations indicates that the effect of increased ejaculation frequency on sperm sex ratios is variable and likely contextual. While causal mechanisms could not be identified, my results demonstrate that sperm sex ratios can vary between multiple ejaculates from the same male. Thus, females may not always receive equal proportions of X- and Y-CBS which confirms the need to consider paternal factors alongside maternal factors in sex allocation research.

Introduction

Hypotheses for the adaptive value of biased offspring sex ratios have predominantly focused on how maternal fitness would be enhanced by adjusting offspring sex ratios according to the relative fitness returns from investment into sons versus daughters (e.g. Charnov *et al.*, 1981; Clark, 1978; Hamilton, 1967; Trivers & Willard, 1973). Several maternal traits and aspects of the maternal environment, such as body condition, social rank, availability of resources, and population density, are linked to offspring sex ratios skews that are consistent with predicted fitness benefits (reviewed by Hardy, 1997; Navara, 2018; West, 2009). In comparison, the contribution of fathers has been relatively under-studied (see Edwards & Cameron, 2014), in part due to the assumption that sex chromosome segregation at meiosis constrains the proportion of X- and Y-chromosome-bearing spermatozoa (CBS) in an ejaculate to parity (e.g. Goldman *et al.*, 1993). However, we now appreciate that mammalian sperm sex ratios are more variable than previously thought (reviewed in Edwards & Cameron, 2014). Further, it is clear that variation in sperm sex ratios can influence offspring sex ratios (e.g. Chandler *et al.*, 2007; Malo *et al.*, 2017; Saragusty *et al.*, 2012).

Although most sex allocation hypotheses have been developed according to maternal fitness benefits (see West, 2009), these theoretical benefits often also apply to fathers. For example, when more attractive males achieve higher fitness and that attractiveness is inherited by sons, both mothers and fathers would benefit from having more sons when the father is attractive (Burley, 1981; Fawcett *et al.*, 2006; Gomendio *et al.*, 2006). As the heterogametic sex, mammalian males might have some control over sperm development and maturation to influence offspring sex ratios according to their own attractiveness or quality. Sperm sex

ratios have been demonstrated to be a plastic trait that likely responds to environmental and social conditions (e.g. Edwards et al., 2017; Lavoie *et al*., 2019; Malo *et al*., 2017). For example, it is hypothesised that mammalian males ejaculate a greater proportion of Y-CBS in response to traits or cues of male attractiveness and quality, such as higher testosterone, fertility, and mating frequency (Edwards & Cameron, 2017; Gomendio *et al.*, 2006; Perret, 2018). If males with higher testosterone, fertility, or mating frequency have greater fitness (e.g. Evans & Simmons, 2008; Gomendio *et al.*, 2000; Harris *et al.*, 1998), such males would be advantaged by siring more sons if these traits are heritable (e.g. Evans & Simmons, 2008; Gomendio *et al.*, 2000; Harris *et al.*, 1998) and confer a greater fitness advantage to their sons than daughters.

While equal numbers of X- and Y-CBS normally result from meiosis, factors that alter spermatogenesis are linked to skewed sperm sex ratios in ejaculates, implying hormonal involvement in the establishment of sperm sex ratios (reviewed in Edwards & Cameron, 2014; James, 2006). For example, in humans, aging, testicular cancer, and exposure to anti-androgenic endocrine-disrupting chemicals lower testosterone regulation of spermatogenesis, resulting in increased genetic and morphological sperm abnormalities and reduced male fertility (Gray *et al.*, 1991; Sharma *et al.*, 2015; Wyrobek *et al.*, 2006; Yeung *et al.*, 2011). These conditions are further linked to greater proportions of X-CBS and daughters (Ansari-Lari & Tanideh, 2009; Gundy *et al.*, 2004; Jacobsen *et al.*, 2000; James, 2006; Møller, 1998; Robbins *et al.*, 2008; Sartorelli *et al.*, 2001; Stone *et al.*, 2013), suggesting that Y-CBS are more vulnerable to abnormalities or damage when spermatogenesis is altered. Indeed, morphological and genetic abnormalities are more prevalent among Y-CBS (Chaudhary *et al.*, 2014; Shi *et al.*, 2019). Conversely, there is evidence from several species to suggest that

males with higher testosterone or increased fertility (associated with higher testosterone in some species; e.g. Malo *et al.*, 2009; Preston *et al.*, 2012) have more sons (red deer (*Cervus elaphus*); Gomendio et al., 2006; humans (*Homo sapiens*); James, 2008; lesser mouse lemur (*Microcebus murinus*); Perret, 2018), suggesting greater proportions or competitiveness of Y-CBS when paternal testosterone is elevated. Thus, pervasive paternal conditions, such as endocrine-disrupting chemicals, may result in preferential loss or damage of X- or Y-CBS, thereby establishing consistently skewed sperm sex ratios in the ejaculates of some males. Moreover, some paternal traits vary temporally, such as seasonal fluctuations in testosterone concentrations (Gerlach & Aurich, 2000) and changes to mating frequency (Chandler *et al.*, 2002), such that sperm sex ratios may vary not only between individual males but also within individuals across time.

The number of studies linking intrinsic and environmental factors to consistent skews in ejaculated sperm sex ratios continues to grow (e.g. Lavoie *et al.*, 2019; Malo *et al.*, 2017; Song *et al.*, 2018; Tiido *et al.*, 2005; Van Hooft *et al.*, 2010; for a review, see Edwards & Cameron, 2014). However, investigations into paternal influences on sex ratios to date have provided a limited picture of paternal sex allocation. While technological advancements have allowed for more accurate assessment of sperm sex ratios (e.g. Edwards *et al.*, 2016; Garner, 2006; Parati *et al.*, 2006; Vanthournout *et al.*, 2018), many recent studies have measured sperm sex ratios from epididymal samples (e.g. Edwards *et al.*, 2019; Edwards *et al.*, 2016; Lavoie *et al.*, 2019; Malo *et al.*, 2017). While results from these studies demonstrate pervasive effects that establish consistent skews in sperm sex ratios (e.g. endocrine-disrupting chemicals, aging, genetic factors), such experimental designs do not account for variation in sperm sex ratios in response to proximal breeding conditions. For example,

changes to mating frequency in domestic cattle (*Bos tarus*) are linked to changes in ejaculated sperm sex ratios (Chandler *et al.*, 2002; Edwards & Cameron, 2017; Hilsenrath *et al.*, 1997), suggesting sperm sex ratios vary between ejaculates of the same male according to the current breeding conditions. Thus, there is a need to also investigate variation in sperm sex ratios at the ejaculate level. While several studies have measured ejaculated sperm sex ratios (e.g. Graffelman *et al.*, 1999; Martin, 1997; Saragusty *et al.*, 2012; Tiido *et al.*, 2005), few have assessed more than a single ejaculate sample (but see Chandler *et al.*, 2002; Chaudhary *et al.*, 2014; DeYoung *et al.*, 2004; Johannisson *et al.*, 2001) and have thus not tested the extent to which ejaculated sperm sex ratios vary within an individual male. More comprehensive investigations are required to determine the extent to which sperm sex ratios vary between ejaculates as breeding conditions change, for example as mating frequency increases.

Variation in male mating frequency in several species has been linked to variation in ejaculated sperm sex ratios, but the nature of this relationship is not yet clear, in part due to limitations of studies to date (e.g. cattle *(Bos tarus);* Chandler *et al.*, 2002; laboratory mice *(Mus musculus);* Edwards & Cameron, 2017; humans *(Homo sapiens);* Hilsenrath *et al.*, 1997). A recent study demonstrated that male mice who had recently mated with several females had more X-CBS remaining in the epididymis compared to males who had recently mated with a single female (Edwards & Cameron, 2017). A potential explanation for these results is that males with a higher mating frequency were ejaculating greater proportions of Y-CBS, thereby leaving more X-CBS in the epididymis (Edwards & Cameron, 2017). By what mechanism differential ejaculation of X- versus Y-CBS from the epididymis would be possible is unknown. However, as the composition of the sperm population in an ejaculate can vary

between subsequent ejaculates (Fitzpatrick & Lüpold, 2014; Perry et al., 2015). It is plausible that similar mechanisms might also facilitate variation in the sperm sex ratio in an ejaculate. Since male mating frequency is a hypothesised cue of male attractiveness in that more attractive males likely mate more often (see Edwards & Cameron, 2014), males may ejaculate a greater proportion of Y-CBS when their mating success is high, and the production of sons could be considered beneficial. However, since the conclusions from Edwards & Cameron (2017) were drawn from epididymal spermatozoa samples, it was not known whether ejaculated sperm sex ratios varied according to the ejaculation order.

Mating frequency might also affect ejaculated sperm sex ratios through differences in viability between the X- and Y-CBS in sperm storage during periods of sexual rest (Hendricks *et al.*, 2008; Hilsenrath *et al.*, 1997). Mature spermatozoa are stored until ejaculation, during which time they lose viability due to accumulated damage from extended exposure to reactive oxygen species (ROS; Jones, 2004; Pizzari *et al.*, 2008). It is hypothesised that X-CBS are more robust to the effects of sperm aging (Hendricks *et al.*, 2008; Lechniak *et al.*, 2003; Oyeyipo *et al.*, 2017; You *et al.*, 2017), in part due to the greater vulnerability of Y-CBS to oxidative damage (Kocer *et al.*, 2015; Oyeyipo *et al.*, 2017; You *et al.*, 2017). Thus, Y-CBS may lose viability more rapidly than X-CBS during periods of sexual rest. If so, post-sexual rest ejaculates may contain a higher proportion of viable X-CBS; as mating frequency increases and spermatozoa showing ROS-related aging effects are depleted, the proportion of viable (but not total) Y-CBS would increase (Amann *et al.*, 1979; Borges Jr. *et al.*, 2019; Gosálvez *et al.*, 2011; Ollero *et al.*, 1996),

Here I assessed the extent to which ejaculated sperm sex ratios vary in relation to ejaculation frequency in the domestic horse (*Equus ferus caballus*). Several studies have shown sex allocation in horses in response to maternal traits (e.g. Cameron & Linklater, 2007; Cameron *et al.*, 1999; Monard *et al.*, 1997; Santos *et al.*, 2015), however, tests of paternal influences on sex ratios in horses have been limited to investigation into paternal age effects on foal sex ratios (Santos *et al.*, 2015). I collected successive ejaculates from two populations of stallions that differed in breed, location, and ejaculation frequency. I increased the ejaculation frequency of all stallions to induce sexual exhaustion and measured the sperm sex ratio (here forth referring to the proportion of X-CBS in the ejaculate) in each successive ejaculate. This experimental design allowed me to measure variation in sperm sex ratios at an individual and ejaculate level. I posed two alternate hypotheses: 1) as ejaculation frequency increases, the total proportion of ejaculated Y-CBS will also increase, reflecting the relationship between mating frequency and male attractiveness; 2) as ejaculation frequency increases and reduces the proportion of aged spermatozoa in each successive ejaculate, the proportion of live (but not total) Y-CBS will increase.

Methods

Population 1: Ellerston stallions

The first population of stallions were five thoroughbred cross (polo pony) stallions from a private stud farm, the Ellerston Polo Club, located approximately 200 km northwest of Newcastle, NSW. They varied in age from five to eight years old. The stallions had full access to pasture and hay. Each stallion was housed alone but within sight of other stallions and

mares. All stallions had been previously conditioned to mount a dummy mare for ejaculate collection into an artificial vagina. As commercial breeding stallions, each stallion had ejaculates collected for artificial insemination purposes nearly every second day between September and late January during the time of this study (see Table 3.1).

Population 2: Newcastle stallions

The second population of stallions were research stallions of the University of Newcastle, agisted in Williamstown, NSW. These five stallions had full access to pasture and hay. Each stallion was housed alone but within sight of other stallions and mares. Four stallions were mixed pony breeds (Shetland and Miniature crossbreds and a Welsh section A) between the ages of 8 and 11; the fifth stallion was a 25-year-old quarter horse. Each stallion had been conditioned to mount a dummy mare and ejaculate into an artificial vagina. The frequency of ejaculate collections in these stallions was more variable and infrequent relative to the Ellerston stallions. The four pony stallions were collected zero to twice per week between October and February during the time of this study, the 25-year-old quarter horse stallion had not been collected from for several years (see Table 3.1).

Ethical Note

All collections conducted in this study adhered to the *Australian Code for the Care and Use of Animals for the Scientific Purposes* and were approved by the University of Tasmania Ethics Committee (Project number: A0015988).

Ejaculate collection schedule

To induce sexual exhaustion and deplete stored spermatozoa, ejaculates were collected from each stallion in both populations seven times within three days (see Amann *et al.*, 1979). Three ejaculate collections occurred on the first day and two on each of the following two days. The first ejaculate collection of the day occurred between 7 am and 9 am, the second occurred between 10 am and 12 pm, and the third (only on the first day) occurred between 3 pm and 4 pm. Within each day, each stallion was given at least a three-hour rest period between ejaculate collections. Ejaculate collections for commercial or other research purposes outside this study (here forth referred to as non-experimental ejaculate collections) did not occur during the three days of experimental ejaculate collections.

This three-day experimental ejaculate collection session was repeated three times in each population of stallions. Ejaculates were collected from the Ellerston stallions in January and February of 2017 and January of 2018 (population 1; Table 3.1). There was a minimum of 20 days between collection sessions, during which time these stallions had ejaculates collected every other day for commercial purposes. Ejaculates were collected from the Newcastle stallions in October, November, and December of 2017 (population 2; Table 3.1). There was also a minimum of 20 days between collection sessions; however, there were fewer non-experimental ejaculate collections that occurred between sessions in this population (0 to 2 within three weeks). This difference resulted in variation in the length of sexual rest periods prior to the experimental collection session between the two populations. The Ellerston stallions had at least two days sexual rest prior to the experimental ejaculate collection session, whereas the Newcastle stallions had at least a week (but up to three weeks) of sexual rest. Due to limitations on stallion availability, not every stallion was available each

month. One of the Ellerston stallions was only available in January 2018 and the 25-year-old Newcastle stallion was unavailable in December 2017.

Table 3.1. Summary of the differences between the two experimental populations of stallions.

	Population 1: Ellerston Stallions (n=5)	Population 2: Newcastle Stallions (n=5)
Location	Ellerston, NSW	Williamstown, NSW
Stallion breeds	Thoroughbred cross	Mixed pony breeds, quarter horse
Stallion ages	5 to 8 years old	8 to 25 years old
Experimental collections	Jan 2017, Feb 2017, Jan 2018	Oct 2017, Nov 2017, Dec 2017
*Frequency of non-experimental collections**	~ 3 times per week	~ 0 to 2 times per week
*Consistency of non-experimental collections**	Consistently	Sporadically
Sexual rest days prior to experiment	~ 2 days	~ 7-20 days
Teaser mare	Naturally in oestrous	Chemically induced oestrous

* during the breeding season

Ejaculate collection and processing

Ejaculate collection followed standard procedures using an artificial vagina, dummy mare, and teaser mare: stallions were sexually excited by the teaser mare, after which they mounted the dummy mare and ejaculated into an artificial vagina. Teaser mares were in oestrous, either naturally or chemically induced (Table 3.1). Teaser mares were kept consistent during ejaculate collection sessions when possible such that the stallion was familiar with the mare across the collection session. However, due to limitations on mare availability or reactions between the mare and stallion (i.e. aggression or lack of interest), it was not possible for teaser mares to be kept consistent across all ejaculate collection sessions. For all stallions, ejaculates were collected using either a pony-size or full-size Missouri artificial vagina (Minitube, VIC, AUS), pre-warmed and lubricated with an inline filter. To quantify sperm depletion, ejaculate volume was recorded, and sperm concentration

was calculated as spermatozoa per mL of ejaculate by either a Nucleocounter ™ SP-100 (ChemoMetec, Allerød, Denmark) or a Spermacue ™ (Minitube, VIC, AUS). The total sperm number in an ejaculate was calculated from the ejaculate volume and sperm concentration.

Spermatozoa preparation

From each ejaculate, 1 mL of raw ejaculate was centrifuged at 400 x *g* for 5 minutes, after which the seminal plasma and spermatozoa were separated and snap-frozen in liquid nitrogen. A sample of spermatozoa from each ejaculate was also cryopreserved: raw ejaculate was diluted 1:1 with semen extender (Minitube, VIC, AUS) and centrifuged at 400 x *g* for 15 minutes. The supernatant, containing the seminal plasma, was discarded and the isolated spermatozoa resuspended in a modified lactose-EDTA cryodiluent (University of Newcastle, NSW, AUS) to a final volume of 2.5 mL. The suspended spermatozoa were loaded into 0.25 mL straws and sealed with polyvinyl alcohol powder (Minitube, VIC, AUS). Straws were placed on a rack and floated 3 cm above liquid nitrogen for ten minutes before being dropped into and stored in liquid nitrogen.

Sperm sex ratios and live:dead analysis via flow cytometry

The protocols for analysing the sex ratio and proportion of live and dead spermatozoa of each spermatozoon were developed following those of Gibb *et al.* (2011) and Aitken *et al*. (2010). Raw ejaculates were diluted in Biggers, Whitten and Whittingham (BWW) medium (Biggers *et al.*, 1971) and centrifuged at 400 x *g* for five minutes. The isolated spermatozoa were then resuspended to a standardized concentration of $100x10^6$ spermatozoa/mL. Two

aliquots from each ejaculate were collected. LIVE/DEAD™ Fixable Red Dead Cell Stain (Thermo Fischer Scientific: L34974) was prepared per manufactures instructions and diluted in phosphate-buffered saline (PBS) to a working concentration of 1:2000. One µL of the stock solution was added to each of the two aliquot of spermatozoa and incubated for 20 minutes at 37 °C, then washed and fixed in 2 % paraformaldehyde (Thermo Fisher Scientific: 28908) in PBS on ice for five minutes. Samples were washed by centrifugation once in PBS and then resuspended in 0.1 M glycine (Sigma Aldrich: G7126) in PBS and kept at 4 °C for up to one week until analysis. In preparation for flow cytometry analysis, samples were washed by centrifugation at 1000 *x g* in PBS and stained with 90 µM Hoechst 33342 (Thermo Fisher Scientific: H1399) for 45 minutes at 37 °C. Samples were washed once more by centrifugation and resuspended in PBS for flow cytometer analysis.

Sperm sex ratios (X-CBS/ total) and the proportion of live and dead spermatozoa were determined with a BD FACS Canto II flow cytometer (Beckman Dickinson Immunocytometry Systems, CA, USA) equipped with 405 nm violet, 488 nm blue and 33 nm red lasers with the standard filter setup. The flow cytometer was quality-control checked using BD™ Cytometer Setup and Tracking Beads. The forward scatter (FSC) signal was used as the trigger signal. The FCS voltage the side scatter (SSC) voltage was adjusted to place the events on a linear scale within a bivariant plot of FSC versus SSC with suitable thresholds set to eliminate smaller particles and noise. To minimize cell coincidence and reduce the coefficient of variation (CV), the flow rate was set to the lowest rate for tube acquisition (nominally 1 µL/second). FSC-height versus FCS-area was used to discriminate coincident events and a total of 10,000 events were recorded per sample. The fluorescence from Hoechst 33342-stained spermatozoa was detected through a 450/50 nm band-pass filter on the violet laser, whereas

the fluorescence of the LIVE/DEAD™ Fixable Red Dead Cell Stain was detected through a 660/20 nm band-pass filter on the red laser. Area and height readings were recorded on a logarithmic scale. Voltages of the populations were determined on the bivariant plot of SSC and Hoechst 33342. Data analysis was performed using FCS Express 6 software. The total, X-CBS, and Y- CBS were gated to obtain the proportion of X- and Y-CBS population in each sample. Inter-assay CV was 3.4. The mean sperm sex ratio of the duplicate samples was used as the final sperm sex ratio value per ejaculate. Accuracy of the flow cytometry was 94 %, as confirmed by qPCR (see below).

Sperm sex ratio analysis via qPCR

To assess the accuracy of the sperm sex ratios measured by flow cytometry, estimates were compared to sperm sex ratios measured by quantitative real-time PCR (qPCR; Joerg *et al.*, 2004; Puglisi *et al.*, 2006) for a subset of ejaculates (n=16). DNA was extracted from one cryopreserved semen straw from each of sixteen ejaculates. Straws were thawed, centrifuged at 500 x *g* for five minutes, then washed by centrifugation with 500 µL of PBS and resuspended in 200 µL of PBS. DNA extraction was performed using DNeasy Blood and Tissue Kit ™ (Qiagen: 69504) following the manufactures instructions. DNA quantity was measured with a Nanodrop 8000™ (Thermo Fisher Scientific).

Sperm sex ratios were determined by the amplification and detection of X- and Y-chromosome specific fragments of the ZFX and SRY genes, respectively, utilising dual labelled Taqman probes. For the X chromosome quantification, forward (5'- CGT AGA AGG AAG TAC TGC AAG AA -3') and reverse (5'- TGG ACT CCC TTG TTT CC -3') primers were used to amplify

a 109 bp fragment on the Equine ZFX gene (GenBank: accession DQ415954.1). A HEX labelled probe (5'-/HEX/ TCA TCT TGA /ZEN/ TTA TAT CTG GCC CAG GAC T/3IABkFQ/-3') was used to detect the resulting PCR products. Y-chromosome quantification used forward (5`- GTC TAG CAG GAC AGC AAC ATA – 3') and reverse (5`- GGC CGT TCT CTC TAC CAT TTC – 3') primers to amplify a 120 bp fragment on the equine sex-determining region of the (Sry) gene (GenBank: accession NM_001081810). Resulting PCR products were detected with a FAM labelled probe (5'- /FAM/TAC TTT GGA/ZEN/CGA GCA ATC CTG GCT /3IABkFQ/-3'). All primers and probes were synthesised by Integrated DNA Technologies (IDT Inc., Coralville, USA).

The qPCR reactions were performed in a total of 10 µL containing 5 µL of PrimeTime Gene Expression Master Mix (IDT Inc: 1055770), 250 nM of each primer, 150 nM of probe, and 2-10 ng of DNA. A Rotorgene-Q ™ (Qiagen) thermal cycler was used for qPCR with the following temperature profile: enzyme activation for three minutes at 95 °C, followed by 40 cycles of 95 °C for 15 seconds, and 60 °C for one minute. Each qPCR experiment included a ten-fold serially diluted standard for each gene run in triplicate alongside triplicate samples and controls. Duplicate technical replicates were run for each qPCR experiment. Starting concentrations were determined for each sample from the threshold cycles calculated for the respective standards. Within each 72 well reaction plate, X- and Y-DNA standard curves (1:6 dilutions; $2E^{-3}$, $2E^{-4}$, $2E^{-5}$, $2E^{-6}$, $2E^{-7}$, $2E^{-8}$ ng of pooled PCR product) were run in triplicate as were all samples and negative controls; replicates that did not fully amplify were excluded. Quantification was determined by the threshold cycles (*Ct*: the number of PCR cycles required for the fluorescence signal to cross a threshold line, which is inversely proportional to the amount of nucleic acid present in the sample) and standard curve from each channel.

The proportion of X-chromosome content in each ejaculate was determined using the following equations (Lavoie *et al.*, 2019; Parati *et al.*, 2006):

$$n = \frac{Ct\ X}{Ct\ Y} \qquad \%X\text{-}CBS = \frac{n}{n+1} \times 100$$

where n corresponds to the relative amounts of X- and Y-chromosomes obtained from *Ct* values. All reaction R^2 and efficiency values were 0.99 and between 0.90 and 1.2 respectively. Inter- and intra-assay variation was quantified using the CV of *Ct* values. Intra-assay CV for X- and Y-DNA was 1.08 and 1.2, respectively. Inter-assay CV for X- and Y-DNA was 7.65 and 11.4, respectively. The mean sperm sex ratio across all replicates of a sample was compared to flow cytometry obtained sperm sex ratio. Sperm sex ratios obtained by flow cytometer were determined to be 94 % accurate by measuring the CV between the qPCR and flow cytometry. All sperm sex ratios are reported from flow cytometry.

Statistical analysis

All analyses were run in R (R Core Team, 2017). As there were several differences between the two populations of stallions (summarised in Table 3.1), analyses were conducted separately for the Ellerston and Newcastle stallions. I measured four aspects of the ejaculate for each of the seven ejaculates collected from each stallion: total sperm number, sperm concentration, ejaculate volume, and sperm sex ratio, and their response to ejaculation frequency, measured as ejaculate number. All variables were transformed for normality. Required transformations were determined using the *transformTukey* function in the *rcompanion* library (Mangiafico, 2016). For Ellerston stallions, total sperm number and ejaculate volume were raised to a power of 0.45, sperm concentration was raised to a power

of 0.58, and sperm sex ratio was log-transformed. For the Newcastle stallions, total sperm number was raised to a power of 0.3, sperm concentration was raised to a power of 0.25, and ejaculate volume was log-transformed.

A linear mixed model (LMM) with the *lmer* function in the *lme4* package (Bates *et al.*, 2014) was fit for each response variable, total sperm number, sperm concentration, ejaculate volume, and sperm sex ratio, to assess the response to ejaculation number. Fixed factors in each of the four LMMs were ejaculate number, month of the ejaculate collection session, and an interaction between the two fixed factors. Stallion ID was included as a random effect to account for repeated measures and provide a measure of inter-stallion variance within populations. Significance of the fixed factors in each LMM was tested using Wald's *t* and F tests with Satterthwaite approximations for degrees of freedom. Model assumptions of heteroscedasticity, normality, and collinearity were tested, and none were violated. Conditional pseudo R^2 was calculated with the *rsquaredGLMM* function in the *MuMIn* package (Barton 2016).

To assess whether variation in sperm sex ratios was related to variation in the viability of X- or Y-CBS during sexual rest, sperm sex ratios among live spermatozoa were compared to the total (live plus dead) sperm sex ratios with a paired t-test from ejaculate collection sessions in January for Ellerston stallions and February for Newcastle stallions. Due to the limited success of fixation of the LIVE/DEAD™ stain in earlier collection sessions, the ratio of live to dead spermatozoa data were only available for these two months. I measured whether the ratio of live to dead spermatozoa changed with ejaculate number by fitting an LMM with the proportion of live spermatozoa as the response variable, ejaculate number as a fixed factor,

and stallion ID as a random effect.

Results

The sperm sex ratio (X-CBS/total) across all ejaculates for the Ellerston stallions ranged from 0.42 to 0.62 and for the Newcastle stallions ranged from 0.49 to 0.69. The mean sperm sex ratio in the first ejaculates collected from the Ellerston stallions, independent to the effect of ejaculation frequency, was 0.49 and ranged from 0.46 to 0.54, whereas the mean sperm sex ratio in the first ejaculates of the Newcastle stallions was 0.59 and ranged from 0.51 to 0.64 (Figure 3.1).

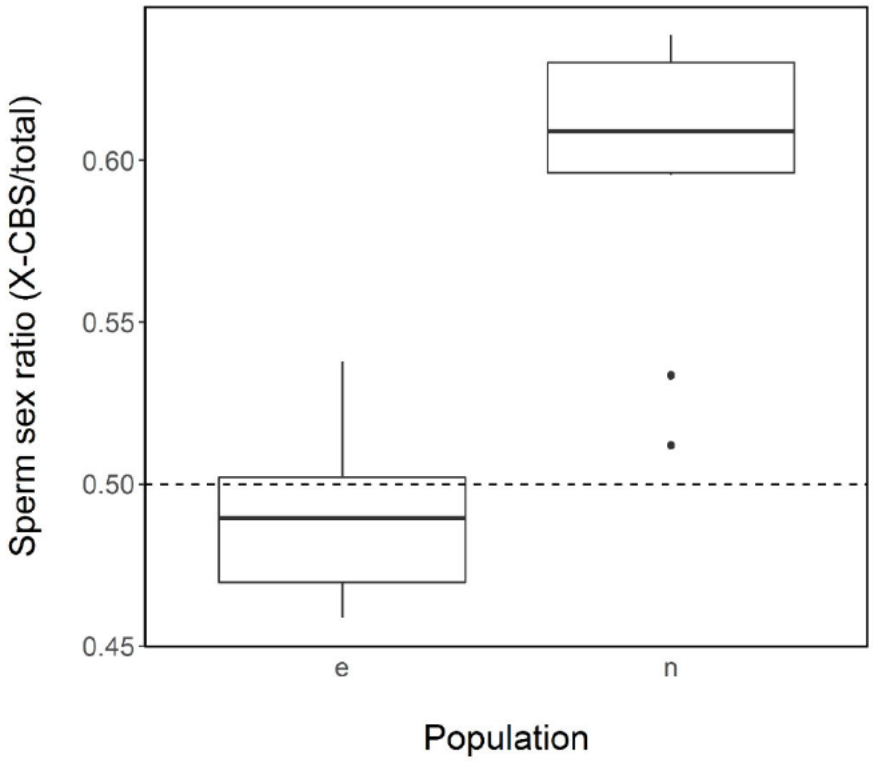

Figure 3.1. The mean sperm sex ratio among the first ejaculates of each ejaculate collection session, independent to the effect of mating frequency, from the Ellerston stallions (n=5) and the Newcastle stallions (n=5). Dashed horizontal line indicates a sperm sex ratio of 50:50.

Population 1: Ellerston stallions

In all months of ejaculate collection sessions, total sperm number and sperm concentration declined with ejaculate number, but ejaculate volume did not vary (Table 3.2; Figure 3.2). Sperm sex ratio increased with ejaculate number in all months of ejaculate collections (Table 3.2; Figure 3.2). Ejaculate number explained 22 % of the variation in sperm sex ratios among stallions (conditional pseudo $R^2 = 0.22$). There was little variation in the sperm sex ratio between individual stallions (LMM: stallion variance = 0.0007 ± 0.02). The proportion of live spermatozoa did not vary with ejaculate number (LMM: $F_{(1, 23)} = 1.77$, Pr(>F) = 0.19) and the sperm sex ratio among live spermatozoa did not differ from the total sperm sex ratio (t = 0.93, df = 27, p|t| = 0.36).

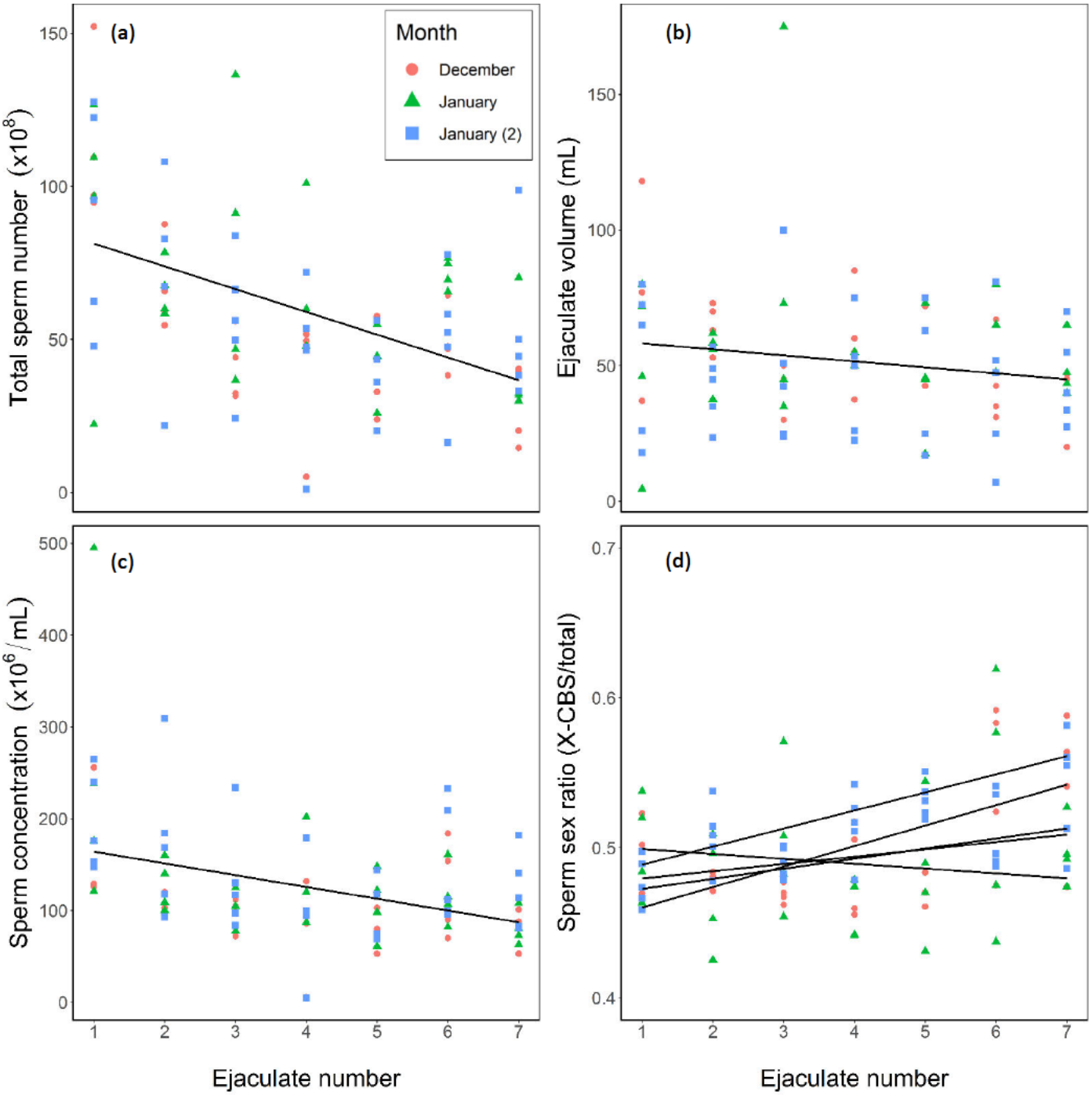

Figure 3.2. Total sperm number (a), ejaculate volume (b), sperm concentration (c), and sperm sex ratio (d) were measured in seven ejaculates (ejaculate number) collected across three days from the Ellerston stallions (n=5). Regression line for each of the five stallions is shown for the relationship between sperm sex ratio and ejaculate number (d). Effects did not differ between the three months of ejaculate collection sessions; the regression line (black) represents effects across all three months of sampling.

Table 3.2. Results of linear mixed models to test whether total sperm number, sperm concentration, ejaculate volume, and sperm sex ratio varied between successive ejaculates (ejaculate number) in the Ellerston stallions (n=5). Significant p-values (>0.05) are in bold.

Fixed factor	F value	df	Pr(>F)
Total sperm number			
ejaculate number	25.89	1, 85	**<.001**
month	0.35	2, 85	0.71
ejaculate number x month	1.09	2, 85	0.34
Sperm concentration			
ejaculate number	17.07	1, 80.9	**<.001**
month	1.65	2, 81.7	0.19
ejaculate number x month	0.65	2, 80.9	0.52
Ejaculate volume			
ejaculate number	2.95	1, 80.1	0.08
month	1.99	2, 80.8	0.14
ejaculate number x month	2.32	2, 80.14	0.11
Sperm sex ratio			
ejaculate number	14.56	1, 81.1	**<.001**
month	0.57	2, 81.9	0.57
ejaculate number x month	1.27	2, 81.8	0.28

Population 2: Newcastle stallions

In all months of ejaculate collection sessions, total sperm number and sperm concentration of the ejaculate declined with ejaculate number; ejaculate volume did not vary (Table 3.3; Figure 3.3). The relationship between ejaculate number and sperm sex ratio differed between all months of ejaculate collection sessions (Table 3.3). There was little variance in the sperm sex ratio between stallions (LMM: stallion variance = 0.00009 ± 0.02). The proportion of live spermatozoa decreased with ejaculate number (LMM: $F_{(1, 22.1)} = 9.16$,

Pr(>F) = 0.006) but there was no difference between the sperm sex ratio among live spermatozoa in comparison to total spermatozoa (t = -1.05, df = 26, p|t| = 0.3).

Table 3.3. Results of linear mixed models to test whether total sperm number, sperm concentration, ejaculate volume, or sperm sex ratio varied between successive ejaculates (ejaculate number) in the Newcastle stallions (n=5). Significant p-values (>0.05) are in bold.

Fixed factor	F value	df	Pr(>F)
Total sperm number			
ejaculate number	57.85	1, 86.9	**<.001**
month	8.29	2, 87.1	**<.001**
ejaculate number x month	1.12	2, 89.9	0.33
Sperm concentration			
ejaculate number	46.1	1, 87.2	**<.001**
month	6.49	2, 87.5	**0.002**
ejaculate number x month	0.85	2, 87	0.45
Ejaculate volume			
ejaculate number	1.23	1, 86.9	0.27
month	1.33	2, 87	0.27
ejaculate number x month	0.85	2, 86.9	0.43
Sperm sex ratio			
ejaculate number	2.03	1, 87.4	0.16
month	2.89	2, 87.9	0.06
ejaculate number x month	3.29	2, 87.4	**0.04**

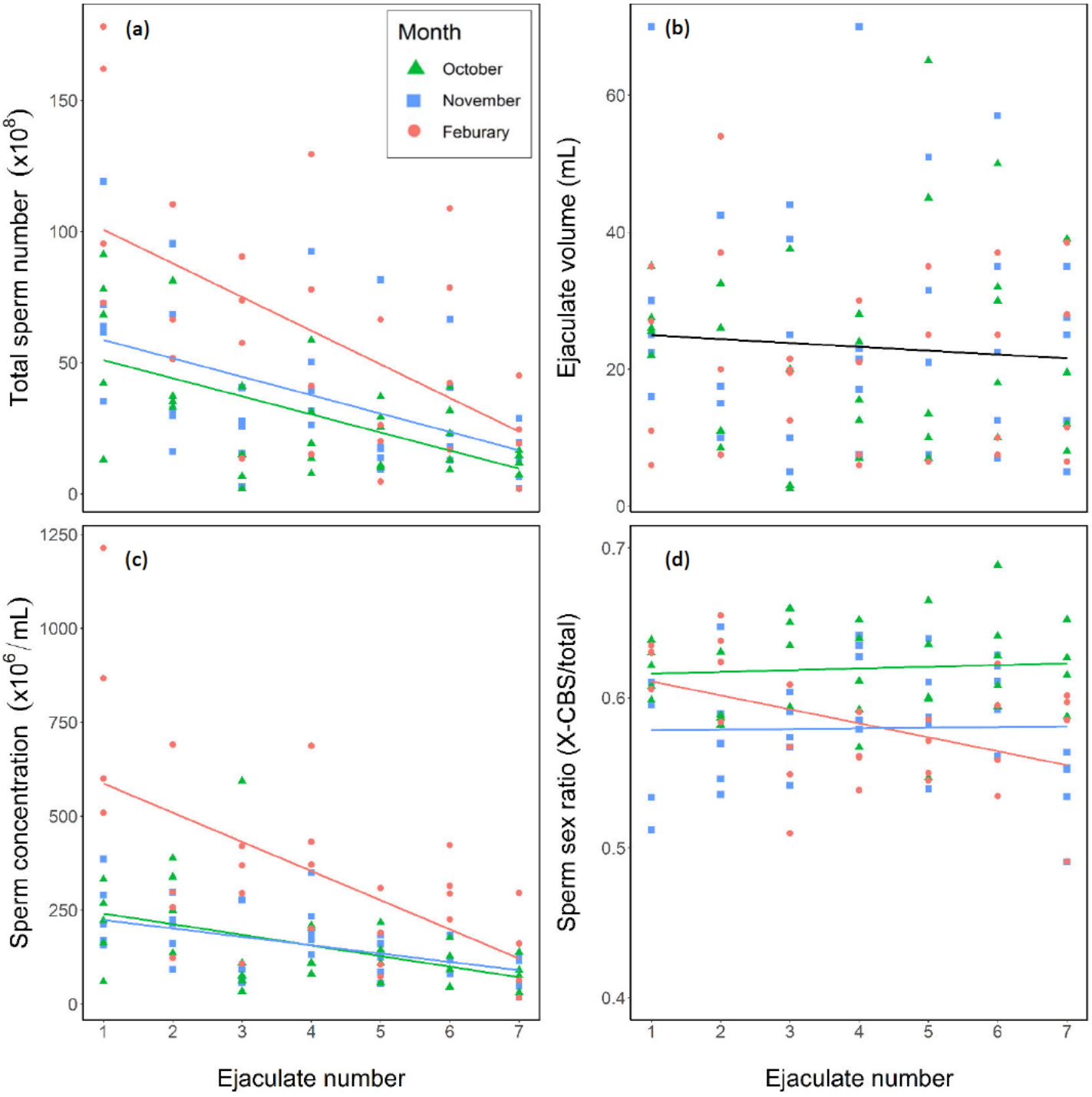

Figure 3.3. Total sperm number (a), ejaculate volume (b), sperm concentration (c), and sperm sex ratio (d) were measured in seven ejaculates collected across three days from the Newcastle stallions (n=5). Effects on total sperm number (a), sperm concentration (c), and sperm sex ratio (d) differed between all three months of ejaculate collection sessions; the regression line for each month is presented. Effects on ejaculate volume (b) did not differ between the three months of ejaculate collection sessions between the three months.

Discussion

I tested whether there was variation in sperm sex ratios at the individual and ejaculate level in two populations of domestic stallions. Sperm sex ratios and the response to increased ejaculation frequency differed in the two populations. Directional ejaculate level variation in sperm sex ratios occurred only among the Ellerston stallions: the proportion of X-CBS increased across successive ejaculates. In the Newcastle stallions, sperm sex ratios remained consistently X-CBS-biased across the successive ejaculates. Practical constraints on the experimental design prevented causal analyses of the difference in results between the two populations, however, these mixed results suggest that the effect of increased ejaculation frequency on ejaculated sperm sex ratios is variable and likely context-dependent. However, it should be noted when interpreting these results, that the sample sizes in this study were limited.

Without further data, only speculative explanations can be offered for the difference in results between the populations. There were some important observations regarding the differences between the two populations that could explain the difference in the relationship between ejaculation frequency and sperm sex ratios. The two populations of stallions differed in the frequency of non-experimental ejaculate collections. The Ellerston stallions, as commercial breeding stallions, were ejaculating nearly every two days throughout the breeding season. In comparison, the Newcastle stallions were ejaculating infrequently and sporadically through the season. This difference in seasonal ejaculation frequency and consistency could have contributed to differences in sperm sex ratios. In other populations of stallions, frequent and consistent exposure to mares and mating opportunities is linked to

higher testosterone concentrations (Khalil *et al.*, 2009; McDonnell, 2000; McDonnell & Murray, 1995). As reduced paternal testosterone is linked to a higher proportion of X-CBS, at least in humans (James, 1994; James, 2008), lower testosterone concentrations relating to the infrequent and sporadic ejaculations of the Newcastle stallions may have contributed to the consistent X-CBS bias. As I did not quantify testosterone concentrations, this explanation is only speculative at this time. However, the X-CBS bias observed among the Newcastle stallions is consistent with the hypothesised links between mating frequency and consistency, testosterone, and the proportion of X-CBS (James, 2008; Khalil *et al.*, 2009; McDonnell & Murray, 1995), thus, further investigation is warranted.

It was not possible to collect ejaculates from each population in the same months: the Ellerston stallions were sampled in December and January while the Newcastle stallions were sampled in October, November, and February. Thus, the differences in sperm sex ratios between the two populations could have related to variation in season. Stallion testosterone concentrations, and ejaculate volume and sperm concentration vary throughout the year (Gerlach & Aurich, 2000; Johnson & Thompson Jr, 1983). Seasonal variation in paternal testosterone concentrations is hypothesised to contribute to seasonal variation in offspring sex ratios (James, 1996). However, it should be noted that seasonal shifts in sperm sex ratios were not observed when measured in men (Chaudhary *et al.*, 2014) or white-tailed deer (*Odocoileus virginianus*; DeYoung *et al.*, 2004). There was some evidence of variation in seasonal effects between the two populations of stallions: the Newcastle stallions, who were collected from both earlier and later in the season, ejaculated higher sperm concentrations relative to the Ellerston stallions, indicative of seasonal variation (Gerlach & Aurich, 2000; Janett *et al.*, 2003; Johnson & Thompson Jr, 1983; Magistrini *et al.*, 1987). Moreover, among

the Newcastle stallions, ejaculates collected in February, the end of the breeding season in the southern hemisphere (Cunningham, 1991), differed from ejaculates collected in October and November with respect to total sperm number, sperm concentration, and sperm sex ratio across successive ejaculates, further suggestive of seasonal variation. However, a more rigorous investigation would be required to assess the contribution of season to variation in sperm sex ratios in domestic stallions.

Additional differences between the two populations included differences in breed and location. The two populations differed in breed in that the Ellerston stallions were all thoroughbred crosses and the Newcastle stallions were mixed pony breeds and one quarter horse. Genetic factors contribute variation in sperm sex ratios (e.g. Malo *et al.*, 2017; Szyda *et al.*, 2000), however, how breed might influence sperm sex ratios would require a more comprehensive investigation, particularly as the breed-variable Newcastle stallions all displayed a consistent X-CBS bias in their ejaculates. The two populations were also geographically distinct, approximately 200 km apart, and may have therefore experienced some differences in environmental conditions. For example, in humans, variation in sperm sex ratios between populations has been attributed to variation in exposure to endocrine-disrupting chemicals in the environment, which can have notable effects on sperm sex ratios (e.g. Kvist *et al.*, 2012; Robbins *et al.*, 2008). Thus, breed traits or variation in environmental conditions cannot be excluded as contributing to the differences in sperm sex ratios between the two populations.

Within each population, there was little variation between stallions in the relationship between sperm sex ratios and ejaculation frequency. However, it should be noted that the number of stallions in each population was small and to more robustly assess individual variation in sperm sex ratios within a population would require a greater sample size. Inter-individual variation in sperm sex ratios within a wider population has been documented in mice (*Mus musculus*; Edwards *et al.*, 2016) and tammar wallabies (*Notamacropus eugenii*; Edwards *et al.*, 2019) and may arise from intrinsic traits such as genetic or epigenetic factors (Malo *et al.*, 2017; Szyda *et al.*, 2000) or differences in age (Sartorelli *et al.*, 2001; Stone *et al.*, 2013).

I had posed two alternate hypotheses predicting ejaculate level variation in sperm sex ratios in response to sexual exhaustion. First, that the total proportion of ejaculated Y-CBS will increase with ejaculation frequency, reflecting the hypothesised relationship between mating frequency and male attractiveness; males with high mating success (and thus also high mating frequency) would benefit from having sons if sons inherit traits associated with high male mating success. Second, that the proportion of live (but not total) Y-CBS will increase with mating frequency due to differential aging effects on the viability of X- and Y-CBS and sexual exhaustion depleting sperm stores. I found little support for either hypothesis; among the Ellerston stallions, the proportion of X-CBS increased across successive ejaculates, whereas among the Newcastle stallions, sperm sex ratios did not vary from an X-CBS bias. Moreover, there were no differences between sperm sex ratios among live versus total sperm sex ratios. I expected the proportion of live spermatozoa to increase in successive ejaculates, however, this was observed only in the Newcastle stallions. These stallions had at least a week, but often longer, of sexual rest prior to experimental ejaculate collections. In

men, a week of sexual rest is linked to a greater proportion of dead spermatozoa in the subsequent ejaculates (Ayad *et al.*, 2018a), consistent with my results. However, there was no evidence of variation in the death rates of X- or Y-CBS during this sexual rest period. Among the Ellerston stallions, there was no change in the proportion of live spermatozoa, suggesting that there was little loss of sperm viability during the two days of sexual rest prior to the experimental ejaculate collections, consistent with observations in men (see Ayad *et al.*, 2018a; Ayad *et al.*, 2018b). It should be noted that, as I measured dead and ruptured spermatozoa, I did not quantify the proportions of dying, damaged, or apoptotic (those programmed for cell death; Aitken & Baker, 2013) X- and Y-CBS. A finer-scale measure of sperm viability might show more subtle effects of sperm aging during sexual rest on X- and Y-CBS.

There also appeared to be little support for the alternative hypothesis that males ejaculate a greater proportion of Y-CBS as ejaculation frequency increases. Rather, there was an increase in the proportion of X-CBS across successive ejaculations of the Ellerston stallions and no change in the sperm sex ratios of the Newcastle stallions. It has been hypothesised that ejaculation frequency might alter sperm sex ratios due to differences in replenishment rates due to intrinsic differences in size between X- and Y-CBS (Edwards & Cameron, 2014). The increase in X-CBS observed here could indicate quicker replenishment of the X-CBS. However, if replenishment rates vary due to inherent differences between the X- and Y-CBS, I would expect to see a similar trend in the Newcastle stallions, even with the initial X-CBS bias, which was not observed. Instead, the shift in sperm sex ratios among the Ellerston stallions could represent a more rapid depletion rate of Y-CBS, reflecting the higher proportion of Y-CBS in the initial experimental ejaculates. The Ellerston stallions were frequently and consistently

ejaculating before each experimental ejaculate collection session and, although sperm sex ratios from these non-experimental ejaculates were not known, as males with perceived high mating success, these stallions could have been ejaculating higher proportions of Y-CBS prior to the experimental collections, thereby leaving more X-CBS in the epididymis (see Edwards & Cameron, 2017). If so, when ejaculation frequency was experimentally increased and spermatozoa depleted, the later experimental ejaculates would contain a greater proportion of X-CBS, as I observed. Interestingly, there was no opposing effect observed among the Newcastle stallions, i.e. later ejaculates did not contain more Y-CBS, despite initial ejaculates being X-CBS-biased, suggesting that the X-CBS bias was not related to or influenced by depletion of epididymal spermatozoa. Although only speculation at this time, the effect of a short-term high ejaculation frequency on ejaculated sperm sex ratios (as tested here) might depend on the seasonal ejaculation frequency. Further investigation into both the effect of seasonal ejaculation frequency and consistency on sperm sex ratios and whether such an effect interacts with a high short-term ejaculation frequency would help to inform this hypothesis.

Conclusions

My results support the growing body of work indicating the role of fathers in mammalian sex allocation (for reviews, see Douhard, 2018; Edwards & Cameron, 2014). Although I could not determine the causal mechanisms of the variation in sperm sex ratios observed here, I showed that ejaculated sperm sex ratios sometimes vary relative to ejaculation frequency. However, the effects of ejaculation frequency on sperm sex ratios were variable, suggesting a more complex relationship than initially predicted and imploring further research. Inter-

ejaculate variation in sperm sex ratios has implications for sex allocation: if males provide unequal proportions of X- and Y-CBS in different ejaculates, males may then influence the sex ratios at fertilization (e.g. Lavoie *et al.*, 2019; Malo *et al.*, 2017; Saragusty *et al.*, 2012). Whether the subtle shift in sperm sex ratios observed here is enough to influence offspring sex ratios is not known. However, other studies confirm that even slight variation in sperm sex ratios influences offspring sex ratios (e.g. Malo *et al.*, 2017; Saragusty *et al.*, 2012). Thus, my results confirm the need to consider paternal factors, including those that drive ejaculate level variation in sperm sex ratios, alongside maternal factors in sex allocation research.

References

Aitken, R. J., & Baker, M. A. (2013). Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. *International Journal of Developmental Biology, 57*, 265-272.

Aitken, R. J., De Iuliis, G. N., Finnie, J. M., Hedges, A., & McLachlan, R. I. (2010). Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Human Reproduction, 25*, 2415-2426.

Amann, R., Thompson, J. D., Squires, E., & Pickett, B. (1979). Effects of age and frequency of ejaculation on sperm production and extragonadal sperm reserves in stallions. *Journal of Reproduction and Fertility Supplement, 27*, 1-6.

Ansari-Lari, M., & Tanideh, N. (2009). Changes in sex hormones and offspring sex ratio following gasoline exposure in male rats. *Comparative Clinical Pathology, 18*, 43-45.

Ayad, B. M., Van der Horst, G., & du Plessis, S. S. (2018a). Revisiting the relationship between the ejaculatory abstinence period and semen characteristics. *International Journal of Fertility and Sterility, 11*, 238-246.

Ayad, B. M., Van der Horst, G., & du Plessis, S. S. (2018b). Short abstinence: a potential strategy for the improvement of sperm quality. *Middle East Fertility Society Journal, 23*, 37-43.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*, 1-48.

Biggers, J., Whitten, W., & Whittingham, D. (1971). The culture of mouse embryos *in vitro*. In J. C. Daniel (Ed.), *Methods in Mammalian Embryology* (pp. 86-94). San Francisco, USA: Freeman & Co. Ltd.

Borges Jr., E., Braga, D., Zanetti, B., Iaconelli Jr., A., & Setti, A. (2019). Revisiting the impact of ejaculatory abstinence on semen quality and intracytoplasmic sperm injection outcomes. *Andrology, 7*, 213-219.

Burley, N. (1981). Sex ratio manipulation and selection for attractiveness. *Science, 211*, 721-722.

Cameron, E. Z., & Linklater, W. L. (2007). Extreme sex ratio variation in relation to change in condition around conception. *Biology Letters, 3*, 395-397.

Cameron, E. Z., Linklater, W. L., Stafford, K. J., & Veltman, C. J. (1999). Birth sex ratios relate to mare condition at conception in Kaimanawa horses. *Behavioral Ecology, 10*, 472-475.

Chandler, J. E., Canal, A. M., Paul, J. B., & Moser, E. B. (2002). Collection frequency affects percent Y-chromosome-bearing sperm, sperm head area and quality of bovine ejaculates. *Theriogenology, 57*, 1327-1346.

Chandler, J. E., Taylor, T. M., Canal, A. L., Cooper, R. K., Moser, E. B., McCormick, M. E., Willard, S. T., Rycroft, H. E., & Gilbert, G. R. (2007). Calving sex ratio as related to the predicted Y-chromosome-bearing spermatozoa ratio in bull ejaculates. *Theriogenology, 67*, 563-571.

Charnov, E. L., Los-den Hartogh, R., Jones, W., & van den Assem, J. (1981). Sex ratio evolution in a variable environment. *Nature, 289*, 27-32.

Chaudhary, I., Jain, M., & Halder, A. (2014). Sperm sex ratio (X: Y Ratio) and its variations. *Austin Journal of Reproductive Medicine & Infertility, 1*, 1-7.

Clark, A. B. (1978). Sex ratio and local resource competition in a prosimian primate. *Science, 201*, 163-165.

DeYoung, R. W., Muller, L. I., Demarais, S., Guthrie, H. D., Welch, G. R., Engelken, T. J., & Gonzales, R. A. (2004). Do *Odocoileus virginianus* males produce Y-chromosome-biased ejaculates? Implications for adaptive sex ratio theories. *Journal of Mammalogy, 85*, 768-773.

Douhard, M. (2018). The role of fathers in mammalian sex allocation. *Mammal Review, 48*, 67-74.

Edwards, A. M., & Cameron, E. Z. (2014). Forgotten fathers: paternal influences on mammalian sex allocation. *Trends in Ecology & Evolution, 29*, 158-164.

Edwards, A. M., & Cameron, E. Z. (2017). Cryptic male choice: experimental evidence of sperm sex ratio and seminal plasma adjustment in relation to coital rate. *Reproduction, Fertility and Development, 29*, 1401-1404.

Edwards, A. M., Cameron, E. Z., Deakin, J. E., Ezaz, T., Pereira, J. C., Ferguson-Smith, M. A., & Robert, K. A. (2019). Sexual conflict in action: an antagonistic relationship between maternal and paternal sex allocation in the tammar wallaby, *Notamacropus eugenii*. *Ecology and Evolution, 9*, 4340-4348.

Edwards, A. M., Cameron, E. Z., Pereira, J. C., & Ferguson-Smith, M. A. (2016). Paternal sex allocation: how variable is the sperm sex ratio? *Journal of Zoology, 299*, 37-41.

Evans, J. P., & Simmons, L. W. (2008). The genetic basis of traits regulating sperm competition and polyandry: can selection favour the evolution of good-and sexy-sperm? *Genetica, 134*, 5-19.

Fawcett, T. W., Kuijper, B., Pen, I., & Weissing, F. J. (2006). Should attractive males have more sons? *Behavioral Ecology, 18*, 71-80.

Fitzpatrick, J. L., & Lüpold, S. (2014). Sexual selection and the evolution of sperm quality. *Molecular Human Reproduction, 20*, 1180-1189.

Garner, D. L. (2006). Flow cytometric sexing of mammalian sperm. *Theriogenology, 65*, 943-957.

Gerlach, T., & Aurich, J. E. (2000). Regulation of seasonal reproductive activity in the stallion, ram and hamster. *Animal Reproduction Science, 58*, 197-213.

Gibb, Z., Morris, L., Maxwell, W., & Grupen, C. (2011). Use of a defined diluent increases the sex-sorting efficiency of stallion sperm. *Theriogenology, 75*, 610-619.

Goldman, A., Fomina, Z., Knights, P., Hill, C., Walker, A., & Hulten, M. (1993). Analysis of the primary sex ratio, sex chromosome aneuploidy and diploidy in human sperm using dual-colour fluorescence *in situ* hybridisation. *European Journal of Human Genetics, 1*, 325-334.

Gomendio, M., Cassinello, J., & Roldan, E. (2000). A comparative study of ejaculate traits in three endangered ungulates with different levels of inbreeding: fluctuating asymmetry as an indicator of reproductive and genetic stress. *Proceedings of the Royal Society of London B: Biological Sciences, 267*, 875-882.

Gomendio, M., Malo, A. F., Soler, A. J., Fernández-Santos, M. R., Esteso, M. C., García, A. J., Roldan, E. R., & Garde, J. (2006). Male fertility and sex ratio at birth in red deer. *Science, 314*, 1445-1447.

Gosálvez, J., González-Martínez, M., López-Fernández, C., Fernández, J. L., & Sánchez-Martín, P. (2011). Shorter abstinence decreases sperm deoxyribonucleic acid fragmentation in ejaculate. *Fertility and Sterility, 96*, 1083-1086.

Graffelman, J., Fugger, E., Keyvanfar, K., & Schulman, J. (1999). Human live birth and sperm-sex ratios compared. *Human Reproduction, 14*, 2917-2920.

Gray, A., Berlin, J. A., McKinlay, J. B., & Longcope, C. (1991). An examination of research design effects on the association of testosterone and male aging: results of a meta-analysis. *Journal of Clinical Epidemiology, 44*, 671-684.

Gundy, S., Babosa, M., Baki, M., & Bodrogi, I. (2004). Increased predisposition to cancer in brothers and offspring of testicular tumor patients. *Pathology Oncology Research, 10*, 197-203.

Hamilton, W. D. (1967). Extraordinary sex ratios. *Science, 156*, 477-488.

Hardy, I. C. (1997). Possible factors influencing vertebrate sex ratios: an introductory overview. *Applied Animal Behaviour Science, 51*, 217-241.

Harris, J. A., Vernon, P. A., & Boomsma, D. I. (1998). The heritability of testosterone: a study of Dutch adolescent twins and their parents. *Behavior Genetics, 28*, 165-171.

Hendricks, K. E., Martins, L., & Hansen, P. J. (2008). Consequences for the bovine embryo of being derived from a spermatozoan subjected to post-ejaculatory aging and heat shock: development to the blastocyst stage and sex ratio. *Journal of Reproduction and Development, 55*, 69-74.

Hilsenrath, R. E., Swarup, M., Bischoff, F. Z., Buster, J. E., & Carson, S. A. (1997). Effect of sexual abstinence on the proportion of X-bearing sperm as assessed by multicolor fluorescent *in situ* hybridization. *Fertility and Sterility, 68*, 510-513.

Jacobsen, R., Bostofte, E., Engholm, G., Hansen, J., Skakkebæk, N. E., & Møller, H. (2000). Fertility and offspring sex ratio of men who develop testicular cancer: a record linkage study. *Human Reproduction, 15*, 1958-1961.

James, W. H. (1994). Sex ratios of the offspring of patients with multiple sclerosis. *Neuroepidemiology, 13*, 216-219.

James, W. H. (1996). Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels at the time of conception. *Journal of Theoretical Biology, 180*, 271-286.

James, W. H. (2006). Offspring sex ratios at birth as markers of paternal endocrine disruption. *Environmental Research, 100*, 77-85.

James, W. H. (2008). Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Journal of Endocrinology, 198*, 3-15.

Janett, F., Thun, R., Bettschen, S., Burger, D., & Hassig, M. (2003). Seasonal changes of semen quality and freezability in Franches-Montagnes stallions. *Animal Reproduction Science, 77*, 213-221.

Joerg, H., Asai, M., Graphodatskaya, D., Janett, F., & Stranzinger, G. (2004). Validating bovine sexed semen samples using quantitative PCR. *Journal of Animal Breeding and Genetics, 121*, 209-215.

Johannisson, R., Leuschner, E., Hüppe, M., Hinrichs, F., Al-Hasani, S., Diedrich, K., Schwinger, E., & Mennicke, K. (2001). Increased frequency of X-bearing sperm in males from an infertility clinic: analysis by two-color fluorescence *in situ* hybridization. *Cytogenetic and Genome Research, 98*, 240-244.

Johnson, L., & Thompson Jr, D. L. (1983). Age-related and seasonal variation in the sertoli cell population, daily sperm production and serum concentrations of follicle-stimulating hormone, luteinizing hormone and testosterone in stallions. *Biology of Reproduction, 29*, 777-789.

Khalil, A. M., Nakahara, K., Tokuriki, M., Kaseda, Y., & Murakami, N. (2009). Variation in fecal testosterone hormone concentration with season and harem size in Misaki feral horses. *Journal of Veterinary Medical Science, 71*, 1075-1078.

Kocer, A., Henry-Berger, J., Noblanc, A., Champroux, A., Pogorelcnik, R., Guiton, R., Janny, L., Pons-Rejraji, H., Saez, F., & Johnson, G. D. (2015). Oxidative DNA damage in mouse sperm chromosomes: size matters. *Free Radical Biology and Medicine, 89*, 993-1002.

Kvist, L., Giwercman, Y. L., Jönsson, B. A., Lindh, C. H., Bonde, J. P., Toft, G., Strucinski, P., Pedersen, H. S., Zvyezday, V., & Giwercman, A. (2012). Serum levels of perfluorinated compounds and sperm Y: X chromosome ratio in two European populations and in Inuit from Greenland. *Reproductive Toxicology, 34*, 644-650.

Lavoie, M. D., Tedeschi, J. N., Garcia-Gonzalez, F., & Firman, R. C. (2019). Exposure to male-dominated environments during development influences sperm sex ratios at sexual maturity. *Evolution Letters, 3*, 392-402.

Lechniak, D., Strabel, T., Bousquet, D., & King, A. (2003). Sperm pre-incubation prior to insemination affects the sex ratio of bovine embryos produced *in vitro*. *Reproduction in Domestic Animals, 38*, 224-227.

Magistrini, M., Chanteloube, P., & Palmer, E. (1987). Influence of season and frequency of ejaculation on production of stallion semen for freezing. *Journal of Reproduction and Fertility Supplement, 35*, 127-133.

Malo, A. F., Martinez-Pastor, F., Garcia-Gonzalez, F., Garde, J., Ballou, J. D., & Lacy, R. C. (2017). A father effect explains sex-ratio bias. *Proceedings of the Royal Society B: Biological Sciences, 284*, 20171159.

Malo, A. F., Roldan, E. R. S., Garde, J. J., Soler, A. J., Vicente, J., Gortazar, C., & Gomendio, M. (2009). What does testosterone do for red deer males? *Proceedings of the Royal Society B: Biological Sciences, 276*, 971-980.

Mangiafico, S. (2016). *Summary and Analysis of Extension Program Evaluation in R, Version 1.18. 1*. North Brunswick, USA: Rutgers Cooperative Extension.

Martin, J. F. (1997). Length of the follicular phase, time of insemination, coital rate and the sex of offspring. *Human Reproduction, 12*, 611-616.

McDonnell, S. M. (2000). Reproductive behavior of stallions and mares: comparison of free-running and domestic in-hand breeding. *Animal Reproduction Science, 60*, 211-219.

McDonnell, S. M., & Murray, S. C. (1995). Bachelor and harem stallion behavior and endocrinology. *Biology of Reproduction, Monograph Series 1*, 577-590.

Møller, H. (1998). Trends in sex-ratio, testicular cancer and male reproductive hazards: are they connected? *Journal of Pathology, Microbiology, and Immunology, 106*, 232-239.

Monard, A. M., Duncan, P., Fritz, H., & Feh, C. (1997). Variations in the birth sex ratio and neonatal mortality in a natural herd of horses. *Behavioral Ecology and Sociobiology, 41*, 243-249.

Navara, K. J. (2018). *Choosing Sexes: Mechanisms and Adaptive Patterns of Sex Allocation in Vertebrates*. New York, USA: Springer.

Ollero, M., Muiño-Blanco, T., López-Pérez, M., & Cebrián-Pérez, J. (1996). Viability of ram spermatozoa in relation to the abstinence period and successive ejaculations. *International Journal of Andrology, 19*, 287-292.

Oyeyipo, I. P., van der Linde, M., & du Plessis, S. S. (2017). Environmental exposure of sperm sex-chromosomes: a gender selection technique. *Toxicological Research, 33*, 315-323.

Parati, K., Bongioni, G., Aleandri, R., & Galli, A. (2006). Sex ratio determination in bovine semen: a new approach by quantitative real time PCR. *Theriogenology, 66*, 2202-2209.

Perret, M. (2018). Revisiting the Trivers-Willard theory on birth sex ratio bias: role of paternal condition in a Malagasy primate. *PLoS One, 13*, e0209640.

Pizzari, T., Dean, R., Pacey, A., Moore, H., & Bonsall, M. B. (2008). The evolutionary ecology of pre- and post-meiotic sperm senescence. *Trends in Ecology & Evolution, 23*, 131-140.

Preston, B. T., Stevenson, I. R., Lincoln, G. A., Monfort, S. L., Pilkington, J. G., & Wilson, K. (2012). Testes size, testosterone production and reproductive behaviour in a natural mammalian mating system. *Journal of Animal Ecology, 81*, 296-305.

Puglisi, R., Vanni, R., Galli, A., Balduzzi, D., Parati, K., Bongioni, G., Crotti, G., Duchi, R., Galli, C., & Lazzari, G. (2006). *In vitro* fertilisation with frozen–thawed bovine sperm sexed by flow cytometry and validated for accuracy by real-time PCR. *Reproduction, 132*, 519-526.

R Core Team (2017). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*. Vienna, Austria.

Robbins, W. A., Wei, F., Elashoff, D. A., Wu, G., Xun, L., & Jia, J. (2008). Y: X sperm ratio in boron-exposed men. *Journal of Andrology, 29*, 115-121.

Santos, M. M., Maia, L. L., Nobre, D. M., Neto, J. F. O., Garcia, T. R., Lage, M. C. G. R., de Melo, M. I. V., Viana, W. S., Palhares, M. S., & da Silva Filho, J. M. (2015). Sex ratio of equine offspring is affected by the ages of the mare and stallion. *Theriogenology, 84*, 1238-1245.

Saragusty, J., Hermes, R., Hofer, H., Bouts, T., Göritz, F., & Hildebrandt, T. B. (2012). Male pygmy hippopotamus influence offspring sex ratio. *Nature Communications, 3*, 1-11.

Sartorelli, E. M. P., Mazzucatto, L. F., & de Pina-Neto, J. M. (2001). Effect of paternal age on human sperm chromosomes. *Fertility and Sterility, 76*, 1119-1123.

Sharma, R., Agarwal, A., Rohra, V. K., Assidi, M., Abu-Elmagd, M., & Turki, R. F. (2015). Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reproductive Biology and Endocrinology, 13*, 1-20.

Shi, X., Chan, D. Y. L., Zhao, M. P., Chan, C. P. S., Huang, J., & Li, T. C. (2019). Comparison of DNA fragmentation levels in spermatozoa with different sex chromosome complements. *Reproductive Biomedicine Online, 38*, 56-65.

Song, W. H., Mohamed, E. A., Pang, W. K., Kang, K. H., Ryu, D. Y., Rahman, M. S., & Pang, M. G. (2018). Effect of endocrine disruptors on the ratio of X and Y chromosome-bearing live spermatozoa. *Reproductive Toxicology, 82*, 10-17.

Stone, B. A., Alex, A., Werlin, L. B., & Marrs, R. P. (2013). Age thresholds for changes in semen parameters in men. *Fertility and Sterility, 100*, 952-958.

Szyda, J., Simianer, H., & Lien, S. (2000). Sex ratio distortion in bovine sperm correlates to recombination in the pseudoautosomal region. *Genetical Research, 75*, 53-59.

Tiido, T., Rignell-Hydbom, A., Jönsson, B., Giwercman, Y. L., Rylander, L., Hagmar, L., & Giwercman, A. (2005). Exposure to persistent organochlorine pollutants associates with human sperm Y: X chromosome ratio. *Human Reproduction, 20*, 1903-1909.

Trivers, R. L., & Willard, D. E. (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science, 179*, 90-92.

Van Hooft, P., Prins, H. H., Getz, W. M., Jolles, A. E., van Wieren, S. E., Greyling, B. J., van Helden, P. D., & Bastos, A. D. (2010). Rainfall-driven sex-ratio genes in African buffalo

suggested by correlations between Y-chromosomal haplotype frequencies and foetal sex ratio. *BMC Evolutionary Biology, 10*, 1-11.

Vanthournout, B., Busck, M. M., Bechsgaard, J., Hendrickx, F., Schramm, A., & Bilde, T. (2018). Male spiders control offspring sex ratio through greater production of female-determining sperm. *Proceedings of the Royal Society B: Biological Sciences, 285*, 20172887.

West, S. (2009). *Sex Allocation*. Princeton, USA: Princeton University Press.

Wyrobek, A. J., Eskenazi, B., Young, S., Arnheim, N., Tiemann-Boege, I., Jabs, E., Glaser, R., Pearson, F., & Evenson, D. (2006). Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proceedings of the National Academy of Sciences, 103*, 9601-9606.

Yeung, B. H., Wan, H. T., Law, A. Y., & Wong, C. K. (2011). Endocrine disrupting chemicals: multiple effects on testicular signaling and spermatogenesis. *Spermatogenesis, 1*, 231-239.

You, Y. A., Kwon, W. S., Saidur Rahman, M., Park, Y. J., Kim, Y. J., & Pang, M. G. (2017). Sex chromosome-dependent differential viability of human spermatozoa during prolonged incubation. *Human Reproduction, 32*, 1183-1191.

Chapter 4: Sperm sex ratios and seminal plasma composition vary with mating frequency in domestic stallions

Abstract

In mammals, sex allocation research has primarily focused on mothers, but recent evidence shows that fathers also influence offspring sex ratios. Mammalian fathers can influence offspring sex ratios by altering the proportion of X- or Y-chromosome-bearing spermatozoa (CBS) in an ejaculate or the composition of the seminal plasma, which supports sperm survival and function. As there are functional differences between the X- and Y-CBS, the concentration of specific seminal plasma components, including testosterone, oestradiol, progesterone, cortisol, glucose, zinc, copper, calcium, and magnesium may differentially affect the competitiveness of X- and Y-CBS. Consequently, the likelihood of fertilisation success for X- and Y-CBS may alter. My previous work showed that the proportion of X-CBS increased with a higher ejaculation frequency in a group of frequently mating domestic stallions (*Equus ferus caballus*). Here, I tested whether seminal plasma composition also co-varied with sperm sex ratios and ejaculation frequency in this same group of stallions. Seminal magnesium declined across successive ejaculates, but was not correlated with sperm sex ratios; no other seminal plasma component varied with ejaculation frequency. Seminal zinc concentrations were positively correlated to the proportion of Y-CBS in the ejaculate, independent of the effect of mating frequency. As magnesium regulates sperm motility and, along with zinc, is involved in seminal antioxidant activity, variation in seminal magnesium or zinc concentrations may differentially affect X- and Y-CBS due to differences in swimming behaviour and vulnerability to oxidative damage. I have provided evidence of variation in

seminal plasma composition and sperm sex ratios in a direction that could influence offspring sex ratios. These results encourage further investigation into the potential role of seminal plasma composition in sex allocation.

Introduction

Sex allocation theory predicts adaptive shifts in offspring sex ratios according to local conditions that predict sex-specific offspring fitness returns (e.g. Clark, 1978; Hamilton, 1967; Trivers & Willard, 1973). Several parental and environmental attributes have been linked to offspring sex ratio skews, including attributes of the father (reviewed in West, 2009). For example, if attractive males have higher fitness and attractive fathers produce more attractive sons, parents would be advantaged by offspring sex ratios skewed toward these attractive sons (Burley, 1981; Fawcett *et al.*, 2006). Such biases have been predominantly explained as a maternal response to male attractiveness, even in species for which males are the heterogametic sex, such as mammals (e.g. Røed *et al.*, 2007). While it is clear that mammalian mothers can adjust sex ratios (e.g. Cameron, 2004), recent evidence demonstrates that mammalian fathers also influence sex ratios (reviewed in Douhard, 2018; Edwards & Cameron, 2014).

Mammalian males can influence offspring sex ratios though biased proportions of X- and Y-CBS within an ejaculate or by altering the relative post-ejaculation performance of the X- and Y-CBS through changes to seminal plasma composition (Cameron *et al.*, 2017). Sperm sex ratios are more variable than previously assumed (reviewed in Edwards & Cameron, 2014)

and ejaculated sperm sex ratios influence offspring sex ratios (e.g. Chandler *et al.*, 2002; Lavoie *et al.*, 2019; Malo *et al.*, 2017; Saragusty *et al.*, 2012). Several paternal factors have been linked to skewed sperm sex ratios, including mating frequency (Edwards & Cameron, 2017; Hilsenrath *et al.*, 1997; Chapter 3), age (Sartorelli *et al.*, 2001; Stone *et al.*, 2013), fertility (Eisenberg *et al.*, 2012; Johannisson *et al.*, 2001), and exposure to endocrine-disrupting chemicals (Robbins *et al.*, 2008; Tiido *et al.*, 2005). In comparison, the potential influence of seminal plasma composition on sperm or offspring sex ratios has rarely been tested, despite high inter-ejaculate variability (but see Edwards & Cameron, 2017). Sperm function is dependent on seminal plasma providing conditions that enhance the viability, motility, and fertility of spermatozoa (Poiani, 2006). Seminal plasma composition varies between ejaculates according to factors such as mating frequency (Edwards & Cameron, 2017; Kaya *et al.*, 2002), the reproductive value of the female (Joseph *et al.*, 2015), and perceived risk of sperm competition (Galvani & Johnstone, 1998; Kilgallon & Simmons, 2005). For example, domestic horse stallions (*Equus ferus caballus*) produce higher quality seminal plasma when the reproductive value of the mare is considered high (Jeannerat *et al.*, 2017; Jeannerat *et al.*, 2018).

The X- and Y-CBS differ morphologically, physiologically, and biochemically such that there is variation in motility and viability (reviewed in Cameron *et al.*, 2017; Li *et al.*, 2016; Rahman & Pang, 2019). Thus, variation in seminal plasma composition might have differential effects on X- and Y-CBS (Table 4.1), potentially altering the likelihood of an X- or Y-CBS fertilising the oocyte. For example, X- and Y-CBS differ in vulnerability to oxidative stress, with Y-CBS showing higher rates of oxidative damage and reduced lifespans when exposed to higher levels of reactive oxygen species (ROS; Aitken & Krausz, 2001; Kocer *et al.*, 2015; Oyeyipo *et*

al., 2017; You *et al.*, 2017). Seminal plasma antioxidants, such as zinc, copper, and magnesium (Table 4.1), protect spermatozoa from ROS, mitigating oxidative damage (Colagar *et al.*, 2009; Garratt *et al.*, 2013; Huang *et al.*, 2000; Omu *et al.*, 2008). Variation between ejaculates in seminal plasma antioxidant capacity (e.g. Koziorowska-Gilun *et al.*, 2011; Mora *et al.*, 2017) might then differentially affect the post-ejaculation viability and longevity of X- and Y-CBS; *in vitro* addition of antioxidants to spermatozoa has a differential protective effects on the viability of X- and Y-CBS (You *et al*., 2017). Several other seminal plasma components are also linked to sex chromosome-specific effects for spermatozoa, including the steroid hormones testosterone, oestradiol, progesterone, and cortisol (see Table 4.1). Furthermore, variation in the seminal ionic environment might also differentially affect the competitiveness of X- and Y-CBS (see Table 4.1). However, this potential role of seminal plasma in sex ratio determination remains to be empirically investigated

Table 4.1. Summary of tested seminal plasma components, their action in the seminal plasma, and the evidence of sex chromosome-specific effects on spermatozoa in mammalian males (unless otherwise specified).

	Action in seminal plasma	Evidence for potential sex chromosome-specific effects
Testosterone: oestradiol	Biomarker for spermatogenetic function and fertility [1, 2] Negatively correlated with sperm quality and fertility [1, 2]	Plasma testosterone negatively correlated with X-CBS and daughters [21, 22, 23, 24] Increases receptivity of the oocyte to Y-CBS [25] Potential variation in response of X- and Y-CBS to seminal hormones [26, 27] Sex-specific effects of seminal testosterone on offspring (*in fowl*) [28]
Progesterone	Sperm membrane receptors regulate intra-cellular Ca influx [3] Regulates motility and fertilization ability [3]	Hypothetically linked to more daughters [21] Potential differences between capacitation ability of X- and Y-CBS [30] Potential variation in response of X- and Y-CBS to seminal hormones [26, 27]
Cortisol	Increases oxidative stress levels [4, 5, 6] Increases damage to spermatozoa [5, 6, 7]	Oxidative stress more damaging to Y-CBS [31, 32, 33, 34] Higher circulating cortisol in males linked to more daughters [29]
Glucose	Energy source for sperm [8, 9, 10]	Differentially expressed glucose metabolism proteins in X- and Y-CBS [35, 36] May then differentially affect motility of X- and Y-CBS
Zinc	ROS scavenger, reducing oxidative stress levels [11, 12] Component in superoxide dismutase (SOD) antioxidant [11, 12]	Oxidative stress more damaging to Y-CBS [31, 32, 33, 34] Low concentrations may then be more damaging for Y-CBS
Copper	Component in SOD antioxidant [11, 12] Toxic to spermatozoa at high levels [13]	Oxidative stress more damaging to Y-CBS [31, 32, 33, 34] Low concentrations may then be more damaging for Y-CBS
Calcium	Main ionic regulator of motility and capacitation [12, 14, 15] Changes sperm membrane potential [15, 16]	Membrane potential of X- versus Y-CBS may affect Ca influx differently [37, 38] Differentially expressed proteins involved in Ca influx in X- and Y-CBS [35, 36] May then differentially affect motility or capacitation of X- and Y-CBS
Magnesium	Regulates motility; antagonist to calcium [12, 17] Involved in antioxidant actions of SOD and glutathione [18, 19, 20]	Membrane potential of X- versus Y-CBS may affect Ca influx differently [37, 38] Differentially expressed proteins involved in Ca influx in X- versus Y-CBS [35, 36] Oxidative stress more damaging to Y-CBS [31, 32, 33, 34]

1. Luboshitzky *et al.*, 2002 2. Zhang *et al.*, 2010 3. Tamburrino *et al.*, 2014 4. Hatamoto *et al.*, 2006 5. Abd-Aziz *et al.*, 2014 6. Min *et al.*, 2016 7. Briggs, 1973 8. Williams & Ford, 2001 9. Miki, 2007 10. Mannowetz *et al.*, 2012 11. Fallah *et al.*, 2018 12. Mirnamniha *et al.*, 2019 13. Roblero *et al.*, 1996 14. Hong *et al.*, 1984 15. Darszon *et al.*, 1999 16. Espinosa & Darszon, 1995 17. Abou-Shakra *et al.*, 1989 18. Chandra *et al.*, 2013 19. Eghbali *et al.*, 2010 20. Stegmayr *et al.*, 1982 21. James, 2008 22. Jacobsen *et al.*, 2000 23. Astolfi *et al.*, 2001 24. Ansari-Lari & Tanideh, 2009 25. Grant & Irwin, 2005 26. Sarkar, 1984 27. Beck *et al.*, 1976 28. Lelono *et al.*, 2019 29. Navara, 2010 30. Madrid-Bury *et al.*, 2003 31. Aitken & Krausz, 2001 32. Kocer *et al.*, 2015 33. Oyeyipo *et al.*, 2017 34. You *et al.*, 2017 35. De Canio *et al.*, 2014 36. Chen *et al.*, 2012 37. Ishijima *et al.*, 1991 38. Esfahani *et al.*, 2016

Seminal plasma-influenced sex allocation may occur if males alter the concentrations of seminal plasma components with hypothesised sex chromosome-specific effects relative to the prevailing breeding conditions. For example, a recent study showed that sperm sex ratios and seminal plasma composition both respond to mating frequency in a direction that could support paternal sex allocation (Edwards & Cameron, 2017). Male mice (*Mus musculus*) that mated with several females had fewer Y-CBS remaining in the epididymis (suggesting more Y-CBS were ejaculated), and further showed a corresponding increase in seminal glucose, compared to males mating with only one female. As there are differentially expressed proteins involved in glucose metabolism that may sex-specifically affect sperm motility or survival (Rahman & Pang, 2019), seminal plasma-influenced sex allocation is implicated (see Edwards & Cameron, 2017). However, the extent to which seminal glucose or other components with potential sex chromosome-specific effects vary between ejaculates in response to hypothesised drivers of sperm sex ratio skews remains untested. Thus, it is unknown whether seminal plasma components with hypothesised sex-specific effects vary between ejaculates to allow for fathers to further influence the sex ratio though seminal plasma composition.

I tested whether seminal plasma components, ejaculation frequency, and sperm sex ratios co-varied in ejaculates collected from domestic stallions (*Equus ferus caballus*). This study followed my previous experiment in which I found that the proportion of X-CBS increased with an increased ejaculation frequency in a group of breeding stallions (Chapter 3). Here I address two follow-up questions with this same group of stallions: first, whether seminal plasma components vary with ejaculation frequency, and second, whether the

concentrations of seminal plasma components are correlated with the sperm sex ratio in an ejaculate. I measured those seminal plasma components with known potential sex chromosome-specific effects: testosterone, oestradiol, progesterone, cortisol, glucose, zinc, copper, calcium, and magnesium (see Table 4.1). The findings of this study will help to inform whether there is variation in seminal plasma composition that would allow fathers an additional route of influence over sex ratios.

Methods

Subjects

This study occurred simultaneously with my previous study (Chapter 3). Five commercial breeding stallions on a private stud farm, the Ellerston Polo Club, approximately 200 km northwest of Newcastle, NSW, were used for this study; these were the same stallions used in Chapter 3. In these stallions, an increase in ejaculation frequency across three days corresponded to an increase in the proportion of X-CBS (Chapter 3). Stallions were thoroughbred crosses and between the ages of five and eight. Each stallion was housed alone within sight of other stallions and mares. As commercial breeding stallions, each stallion had ejaculates routinely collected from approximately four times per week from September to late January for commercial artificial insemination purposes.

Ejaculate collection and processing

Ejaculates were collected seven times from each stallion within three days in January. Following two days of sexual rest, three ejaculate collections occurred on the first day and two on each of the following two days. The first set of ejaculate collections of the day occurred between 7:00 and 7:30 am, the second between 10:00 and 10:30 am, and the third (only on the first day) between 3:00 and 3:30 pm. Stallions were given three hours rest between each ejaculate collection per day. Ejaculates were collected using a full-size Missouri artificial vagina (Minitube, VIC, AUS), pre-warmed and lubricated with an inline filter, and the presence of a teaser mare naturally in oestrous. The teaser mare was kept as consistent as possible so that the stallion was familiar with the mare. The volume of each ejaculate was recorded. Sperm concentration was calculated as spermatozoa per mL of ejaculate by a Spermacue ™ (Minitube, VIC, AUS). From the ejaculate volume and sperm concentration, the total sperm number in the ejaculate was calculated.

Sperm sex ratio assessment

Sperm sex ratios (hereafter referring to the proportion of X-CBS in the ejaculate) were recorded for each ejaculate using flow cytometry, as described in Chapter 3. Briefly, staining and fixation protocols were developed following Gibb *et al.* (2011) and Aitken *et al.* (2010). The proportion of X-CBS for each ejaculate was recorded by identifying the X- and Y-CBS populations per ejaculate with a FACSCanto II flow cytometer (Beckman Dickinson Immunocytometry Systems, San Jose, CA, USA).

Hormone assays

For sample extraction, 1 mL of seminal plasma was deproteinized in 3 mL of AR grade absolute ethanol (Merck Millipore, AUS: 107017) and vortex-mixed for 1 minute. Samples were then snap-frozen in dry ice-cooled methanol. The ethanolic phase was transferred to a clean tube and evaporated under a stream of nitrogen. Samples were resuspended in 500 µL Milli-Q water (Merck Millipore, AUS) and extracted three times with ethyl acetate (Sigma-Aldrich: 58958). The ethyl acetate was then concentrated to 1.5 mL under a stream of nitrogen and stored at -20 °C until analysis.

Duplicate aliquots of extract (150 µL) of ethyl acetate were evaporated to dryness and resuspended in phosphate-buffered saline-gelatine. Aliquots were then incubated overnight at 4°C with 2,4,6,7 ^{3}H-oestradiol, 1,2,6,7 ^{3}H-testosterone, 1,2,6,7 ^{3}H-progesterone, or 1,2,6,7 ^{3}H-cortisol (NET317250UC, NET370250UC, NET381250UC, NET396250UC respectively PerkinElmer, Victoria, AUS) and testosterone, oestradiol, progesterone, or cortisol antiserum (Novus Biologicals, testosterone: NBP1-78562, oestradiol: NBP1-78621, progesterone: NBP2-45210, cortisol: NB100-62484) in phosphate-buffered saline-gelatine. The standard curve ranged from 3.125 to 800 pg of authentic testosterone, oestradiol, progesterone, and from 12.5 to 800 pg for cortisol. The sensitivity was 1.25 pg for testosterone, oestradiol, and progesterone, and 6.25 pg for cortisol. The intra-assay coefficient of variation was <10 % for all hormones.

For glucose analysis, 100 µL of seminal plasma was deproteinized by mixing with 200 µL of 0.3N barium hydroxide (Sigma-Aldrich: B4059) and 200 µL 0.3N zinc sulphate (Sigma-Aldrich: Z2876). Samples were then centrifuged at 1000 x *g* for 5 minutes and the supernatant

retained. The supernatant was analysed for glucose by scaling down reagent volumes of the Glucose Assay Kit (Sigma-Aldrich: GAHK20) and following kit instructions. Sample volumes ranged from 0.7 to 14 µL; reagent volume was adjusted from 1000 µL to 70 µL. The standard curve ranged from 0.5 to 50 µg of glucose and, when necessary, samples were diluted until within range of the standard curve. Samples were then read at 340 nm on a Varain Carey 50 UV-Vis spectrophotometer (Agilent Technologies Australia Pty. Ltd., VIC, AUS).

Major cation and trace element analysis

Seminal calcium, magnesium, zinc, and copper analyses were performed by Queensland Health Forensic and Scientific Services as detailed in Carter *et al.* (2015). Samples of seminal plasma were weighed (approx. 0.5 g) into digestion tubes. Concentrated nitric acid (10 mL, 70 %; Seastar Chemicals, Canada) was added to each sample at room temperature and heated on a heating mantle set at 90 °C until digested. The digested solutions were made up to 40 mL with Milli-Q water (Merck Millipore, AUS) and then analysed using a 7000 Inductively Coupled Plasma (ICP)-MS and 700 ICP-OES (Agilent Technologies Australia Pty. Ltd., VIC, AUS). Multi-element and single element stock standard solutions (10 mg/L; CHOICE Analytical, NSW, AUS) were used to calibrate solutions of 0, 0.1, 1, 10, 100, and 1000 µg/L in 5 % nitric acid for the screening of each element.

Statistical analysis

All analyses were run in R (R Core Team, 2017). Three sets of analyses were performed. The first set tested the effects of ejaculation frequency on ejaculate traits: total sperm number, sperm concentration, ejaculate volume, sperm sex ratio (Table 4.2.1). The second set tested

the effects of ejaculation frequency on each measured seminal plasma component (Table 4.2.2). The third set evaluated the relationships between each seminal plasma component and the sperm sex ratio in each ejaculate (Table 4.2.3). All tests were performed with linear mixed models (LMMs) fit with the *lmer* function in the *lme4* package (Bates *et al.*, 2014). For each model, assumptions of heteroscedasticity, normality, and collinearity were assessed; none were violated. Significance of fixed factors for all models was determined by F-tests and Wald statistics. P-values were adjusted with a false discover rate (FDR) correction to account for the number of tests (n=20) (Benjamini & Hochberg, 1995). The ratio of seminal testosterone to oestradiol was used in all analyses because the ratio of these two hormones is a more powerful indicator of the hormonal conditions during spermatogenesis (Müller *et al.*, 2012; Zhang *et al.*, 2010). For the second set of models, zinc and calcium were log-transformed and cortisol was raised to the power of 0.225 for normality, as indicated by the *transformTukey* function in the *rcompanion* package (Mangiafico, 2016). In the third set of models to assess the relationship between seminal plasma components and sperm sex ratios, ejaculate number was included as a fixed factor to account for the variation in sperm sex ratios attributed to ejaculation frequency. Stallion identity was included in all models in the three set of tests to account for individual variation between stallions and the repeated measures from each stallion.

Table 4.2. Summary of LMMs testing whether 1) ejaculate traits or 2) seminal plasma components varied with ejaculation frequency (measured as ejaculate number), and 3) whether the concentrations of seminal plasma components related to the sperm sex ratio in each of the seven successive ejaculates collected over three days from five breeding stallions.

			Factors	
Test #	Model #	Dependent	Fixed	Random
1	1.1	*total sperm number*	*ejaculate number*	*stallion ID*
	1.2	*sperm concentration*	*ejaculate number*	*stallion ID*
	1.3	*ejaculate volume*	*ejaculate number*	*stallion ID*
	1.4	*sperm sex ratios*	*ejaculate number*	*stallion ID*
2	2.1	*testosterone:oestradiol*	*ejaculate number*	*stallion ID*
	2.2	*progesterone*	*ejaculate number*	*stallion ID*
	2.3	*cortisol*	*ejaculate number*	*stallion ID*
	2.4	*glucose*	*ejaculate number*	*stallion ID*
	2.5	*zinc*	*ejaculate number*	*stallion ID*
	2.6	*copper*	*ejaculate number*	*stallion ID*
	2.7	*calcium*	*ejaculate number*	*stallion ID*
	2.8	*magnesium*	*ejaculate number*	*stallion ID*
3	3.1	*sperm sex ratio*	*testosterone:oestradiol + ejaculate number*	*stallion ID*
	3.2	*sperm sex ratio*	*progesterone + ejaculate number*	*stallion ID*
	3.3	*sperm sex ratio*	*cortisol + ejaculate number*	*stallion ID*
	3.4	*sperm sex ratio*	*glucose + ejaculate number*	*stallion ID*
	3.5	*sperm sex ratio*	*zinc + ejaculate number*	*stallion ID*
	3.6	*sperm sex ratio*	*copper + ejaculate number*	*stallion ID*
	3.7	*sperm sex ratio*	*calcium + ejaculate number*	*stallion ID*
	3.8	*sperm sex ratio*	*magnesium + ejaculate number*	*stallion ID*

Results

The first set of analysis testing the effects of ejaculation frequency (measured as ejaculate number) on ejaculate traits showed a decline in the total sperm number and sperm concentration across successive ejaculates, but no change in ejaculate volume (Table 4.3.1; Figure 4.1). The proportion of X-CBS in the ejaculate increased with ejaculate number (Table 4.3.1; Figure 4.1).

The second set of analyses of the effects of ejaculation frequency on seminal plasma components showed little directional variation in seminal plasma components across successive ejaculates (Table 4.3.2; Figure 4.2). Only seminal magnesium concentrations declined in successive ejaculates (Figure 4.2.2). Descriptive statistics of the concentrations of each seminal plasma component are presented in Table 4.4.

Results from the third set of analyses testing for a relationship between the concentration of seminal plasma components and the sperm sex ratio in an ejaculate showed a positive association between seminal zinc concentrations and the proportion of Y-CBS (Table 4.3.3; Figure 4.3). However, seminal zinc did not correlate with ejaculation frequency. No other seminal plasma components were associated with sperm sex ratios (Table 4.3.3).

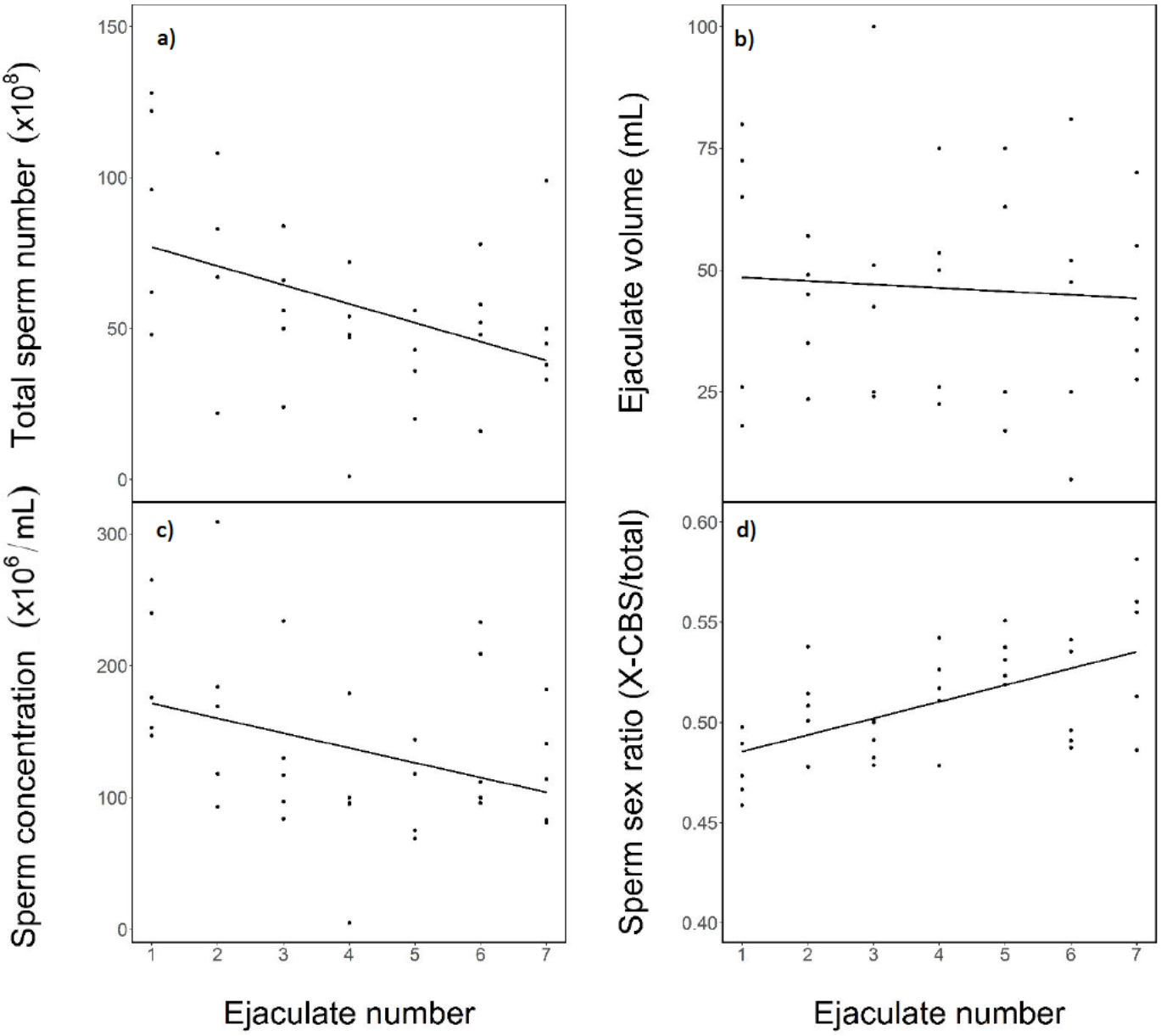

Figure 4.1. Changes to (a) total sperm number, (b) ejaculate volume, (c) sperm concentration, and (d) sperm sex ratio across seven successive ejaculates (ejaculate number) collected during three days from five breeding stallions.

Table 4.3. Results of LMMs testing whether 1) ejaculate traits or 2) seminal plasma components varied with ejaculation frequency (measured as ejaculate number), and 3) whether the concentration of seminal plasma components related to the sperm sex ratio in each of the seven successive ejaculates collected over three days from five breeding stallions.

Test #	Model #	Dependent factor	Fixed factor	F value	df	Pr(>F) [a]	Random variance	n [b]
1	1.1	*total sperm number*	*ejaculate number*	8.72	1, 30	**0.04**	40.5±6.4	35
	1.2	*sperm concentration*	*ejaculate number*	0.28	1, 30	0.69	234.4±15.31	35
	1.3	*ejaculate volume*	*ejaculate number*	6.75	1, 30	**0.04**	17.5±4.2	35
	1.4	*sperm sex ratios*	*ejaculate number*	24.15	1, 30	**>0.001**	1.5±1.2	35
2	2.1	*testosterone:oestradiol*	*ejaculate number*	0.04	1, 34	0.92	0	34
	2.2	*progesterone*	*ejaculate number*	1.15	1, 29.1	0.57	133.3±11.5	34
	2.3	*cortisol*	*ejaculate number*	0.90	1, 27.9	0.57	0.01±0.1	32
	2.4	*glucose*	*ejaculate number*	0.23	1, 35	0.52	0	35
	2.5	*zinc*	*ejaculate number*	0.30	1, 29	0.69	0.1±0.3	35
	2.6	*copper*	*ejaculate number*	2.92	1, 30	0.23	0	35
	2.7	*calcium*	*ejaculate number*	0.30	1, 29	0.69	0.1±0.3	35
	2.8	*magnesium*	*ejaculate number*	7.54	1, 30	**0.04**	0.02±0.1	35
3	3.1	*sperm sex ratio*	*testosterone:oestradiol*	0.01	1, 29.8	0.92	1.5±1.2	34
	3.2	*sperm sex ratio*	*progesterone*	3.02	1, 33.8	0.23	1.2±1.1	34
	3.3	*sperm sex ratio*	*cortisol*	0.02	1, 30.8	0.92	1.3±1.2	32
	3.4	*sperm sex ratio*	*glucose*	0.35	1, 33	0.69	1.5±1.2	35
	3.5	*sperm sex ratio*	*zinc*	19.5	1, 35	**>0.001**	0	35
	3.6	*sperm sex ratio*	*copper*	3.9	1, 34.9	0.17	0.8±0.9	35
	3.7	*sperm sex ratio*	*calcium*	2.2	1, 34.6	0.33	0.9±0.9	35
	3.8	*sperm sex ratio*	*magnesium*	0.9	1, 33.4	0.47	1.1±1	35

[a] FDR correction for multiple testing

[b] some ejaculates did not contain sufficient seminal plasma to perform all hormone analyses, thus the differing sample sizes

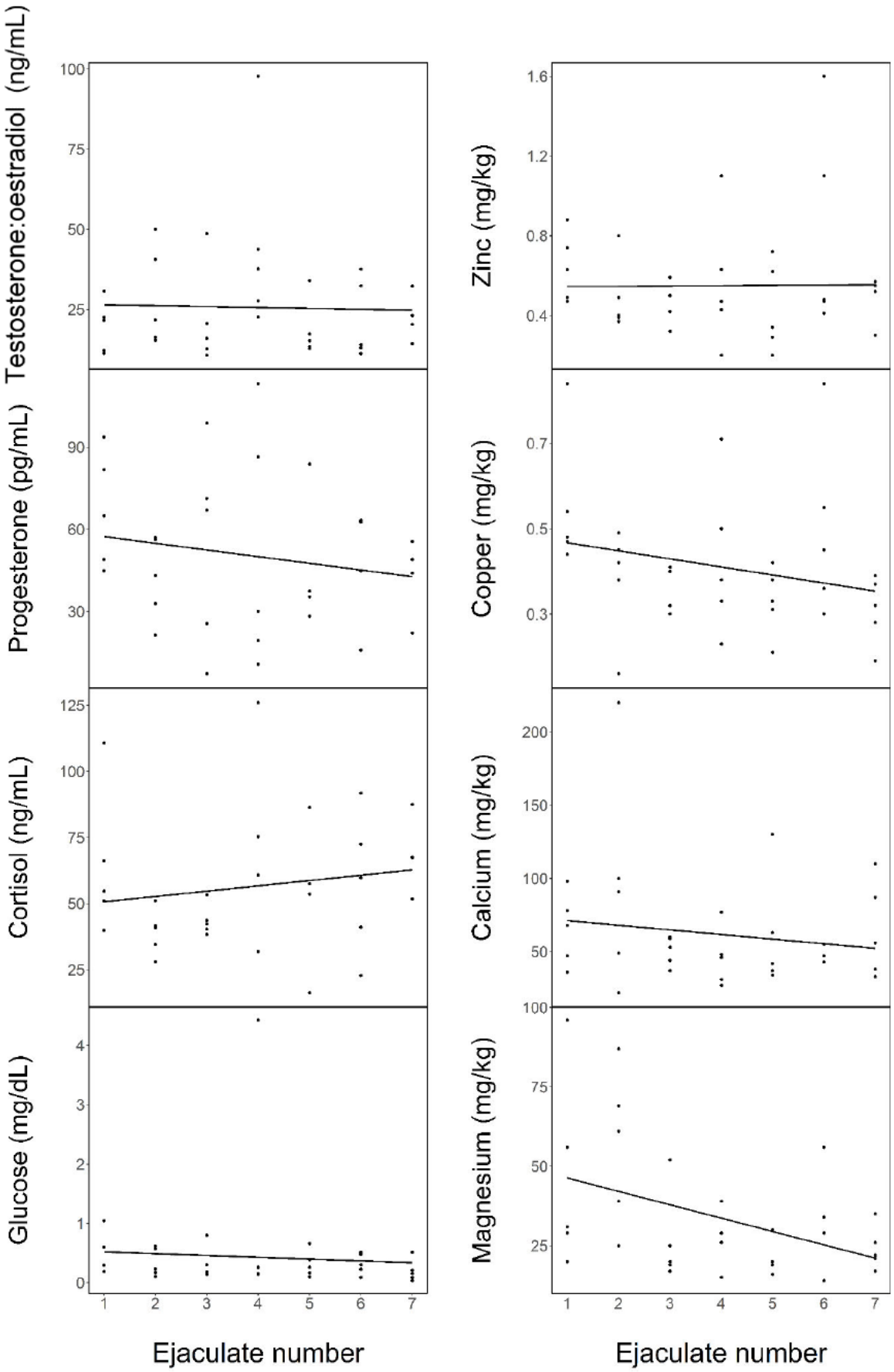

Figure 4.2. The concentrations of each seminal plasma component across seven successive ejaculates (ejaculate number) collected during a three-day period from five breeding stallions.

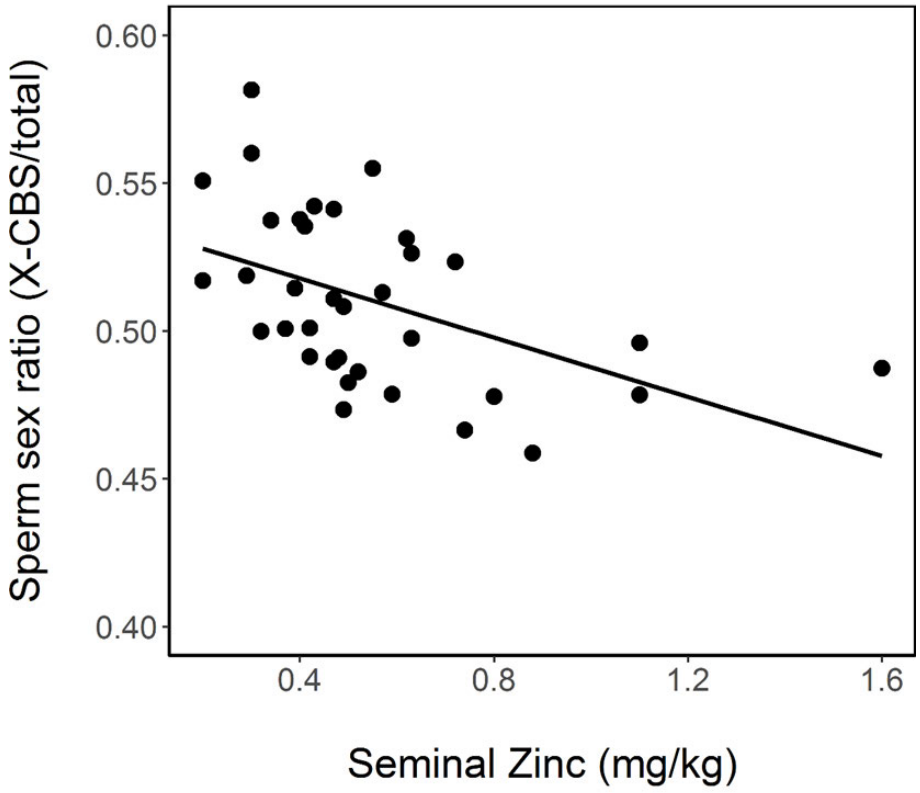

Figure 4.3. Seminal zinc concentrations were negatively associated with the proportion of X-CBS in ejaculates collected from five domestic breeding stallions during a three-day period.

Table 4.4. Descriptive statistics of the concentrations of seminal plasma components from successive ejaculates collected from five domestic breeding stallions during a three-day period.

Seminal plasma component	mean (SD)	min	max
Testosterone:oestradiol (pg/mL)	25.6 ± 17.1	10.67	97.76
Progesterone (pg/mL)	50.21 ± 26.49	7.26	113.16
Cortisol (ng/mL)	56.45 ± 24.45	16.3	126
Glucose (mg/dL)	7.71 ± 13.2	0.63	79.6
Zinc (mg/kg)	0.55 ± 0.28	0.2	1.6
Copper (mg/kg)	0.41 ± 0.15	0.16	0.84
Calcium (mg/kg)	61.71 ± 37.3	22	220
Magnesium (mg/kg)	33.69 ± 19.89	14	96

Discussion

I tested whether seminal plasma composition and sperm sex ratios co-varied across successive ejaculates to assess the potential for fathers to influence offspring sex ratios through seminal plasma composition. Variation in seminal plasma constituents with hypothesised sex-specific effects could provide a route for paternal influence on offspring sex ratios in addition to variation in the proportion of X- and Y-CBS. I found that sperm sex ratios varied in response to ejaculation frequency, with the proportion of X-CBS increasing across successive ejaculates. The total sperm number and sperm concentration also declined, indicative of sperm depletion, however, ejaculate volume show no evidence of depletion. These results agree with findings in my earlier work and suggest a difference in the depletion rates of X- and Y-CBS (Chapter 3). Although my experimental design did not allow for causal analysis, the higher proportion of X-CBS in later ejaculates could represent a more rapid decline in Y-CBS due to the higher proportion of Y-CBS in early ejaculates (see Chapter 3 for further discussion). However, it should be noted that these results were observed in a limited sample size and more rigorous testing with a wider population of stallions should be conducted to further explore the findings of this chapter.

There was limited evidence that seminal plasma composition varied with ejaculation frequency, despite sperm sex ratio variation with ejaculation frequency. There were no increases in the concentrations of any seminal plasma component in the later ejaculates that contained lower proportions of Y-CBS contrary to hypotheses that seminal plasma composition, specifically seminal glucose, might vary in response to the depletion of Y-CBS under increased ejaculation frequency (see Edwards & Cameron, 2017). There was also no

evidence of depletion of any seminal plasma component other than seminal magnesium, which declined across successive ejaculates. In stallions, a portion of seminal magnesium is derived from the caudal epididymis and the ampullary gland (Kareskoski & Katila, 2008). These are the major sperm storage structures and are the first to be depleted by successive ejaculations (Barth, 2007; Segabinazzi *et al.*, 2018). As there was evidence of sperm depletion, the decline in seminal magnesium across successive ejaculates could have related to the depletion of the caudal epididymis and ampullary gland (see Kareskoski & Katila, 2008). However, there was no association between seminal magnesium and sperm sex ratios, despite both the seminal magnesium concentration and proportion of X-CBS in the ejaculate declined with increased ejaculation frequency.

The potential functional consequences of a decline in seminal magnesium on X- and Y-CBS were beyond the scope of this study. However, as an important regulatory ion in both sperm motility (Abou-Shakra *et al.*, 1989) and seminal antioxidant activities (Hemachand & Shaha, 2003; Peeker *et al.*, 1997), changes to seminal magnesium concentrations may have differential effects on the motility or viability of the X- and Y-CBS due to the differences in swimming behaviour (Balli *et al.*, 2004; Penfold *et al.*, 1998; Sarkar, 1984) and resistance to oxidative damage (Aitken & Krausz, 2001; Kocer *et al.*, 2015; Oyeyipo *et al.*, 2017; You *et al.*, 2017). For example, as Y-CBS are more vulnerable to oxidative damage, the depletion of seminal magnesium could have a more negative impact on Y-CBS due to the reduced antioxidant activity (Aitken & Krausz, 2001; Kocer *et al.*, 2015; Oyeyipo *et al.*, 2017; You *et al.*, 2017). Seminal antioxidant capacity and ROS were not quantified in the present study, but other studies have demonstrated a link between reduced seminal magnesium and reduced seminal antioxidant capacity (Abdul-Rasheed, 2010; Chandra *et al.*, 2013; Eghbali *et al.*, 2010;

Tvrdá *et al.*, 2013). Seminal magnesium was lowest in later ejaculates; these ejaculates also contained fewer Y-CBS. Thus, the combined effect of ejaculation frequency on sperm sex ratios and seminal plasma magnesium could increase the likelihood of daughters being conceived when mating frequency is increased to induce sexual exhaustion; this hypothesis requires further investigation.

The seminal zinc concentration in an ejaculate was negatively correlated with the proportion of X-CBS. Zinc is an important antioxidant in seminal plasma, acting both as a ROS scavenger and one of the main components in the antioxidant enzyme SOD (Colagar *et al.*, 2009; Fallah *et al.*, 2018; Garratt *et al.*, 2013; Talevi *et al.*, 2013). Previous studies have linked lower seminal zinc concentrations to reduced antioxidant activity, elevated ROS, and increased sperm DNA damage (Colagar *et al.*, 2009; Garratt *et al.*, 2013; Huang *et al.*, 2000; Omu *et al.*, 2008). Whether the positive association between seminal zinc and sperm sex ratios indicates more antioxidant resources in ejaculates with a greater proportion of Y-CBS cannot be determined from this data. However, males of other taxa do differentially allocate seminal antioxidant resources, specifically SOD, according to the proximate mating conditions (Mora *et al.*, 2017). As the less resistant sperm-type to oxidative stress (Aitken & Krausz, 2001; Kocer *et al.*, 2015; Oyeyipo *et al.*, 2017; You *et al.*, 2017), greater seminal antioxidant activity potentially improves the likelihood of the Y-CBS successfully achieving fertilization. Future work is required to assess this hypothesis. Furthermore, zinc and magnesium are only two of the many antioxidants in seminal plasma; total seminal antioxidant capacity is the sum of several antioxidants, including catalase, glutathione peroxidase, ascorbate, tocopherol (Hammadeh & Hamad, 2009; Mahfouz *et al.*, 2009; Sharma *et al.*, 1999). A measure of total antioxidant capacity of an ejaculate relative to the proportion of Y-CBS and subsequent

viability and longevity of Y-CBS would provide further insight into the potential role of seminal antioxidants in sex allocation.

Conclusions

I found some evidence of variation in seminal plasma composition relative to sperm sex ratios in a direction that could support paternal sex allocation. These results encourage further investigation into the potential role of seminal plasma composition in paternal sex allocation, in particular, whether there are sex chromosome-specific responses of the X- and Y-CBS to oxidative stress exposure in the female reproductive tract and antioxidant activity of the seminal plasma that can ultimately influence the sex of the offspring. I did not test whether the seminal plasma components measured here have sex chromosome-specific effects on the function of the X- or Y-CBS, e.g. motility or fertility. There are several differences between the X- and Y-CBS that plausibly support differential responses in motility or fertility according to the seminal environment (e.g. Oyeyipo *et al.*, 2017; Sarkar, 1984; Van Dyk *et al.*, 2001). If seminal plasma composition differentially affects the motility or fertility of X- and Y-CBS in the female reproductive tract, the probability of an X- or Y-CBS successfully fertilising the oocyte would then differ, thereby altering the probability of conception towards one sex. Future work should test the more subtle effects of seminal plasma composition on the function of the X- and Y-CBS, such as differential effects on motility and fertility.

References

Abd-Aziz, N., Chatterjee, A., Chatterjee, R., & Durairajanayagam, D. (2014). Corticosterone-induced oxidative stress alters epididymal sperm fertility in rats. *ASM Science Journal, 8*, 117-124.

Abdul-Rasheed, O. F. (2010). Association between seminal plasma copper and magnesium levels with oxidative stress in Iraqi infertile men. *Oman Medical Journal, 25*, 168-172.

Abou-Shakra, F. R., Ward, N. I., & Everard, D. M. (1989). The role of trace elements in male infertility. *Fertility and Sterility, 52*, 307-310.

Aitken, R. J., De Iuliis, G. N., Finnie, J. M., Hedges, A., & McLachlan, R. I. (2010). Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Human Reproduction, 25*, 2415-2426.

Aitken, R. J., & Krausz, C. (2001). Oxidative stress, DNA damage and the Y chromosome. *Reproduction, 122*, 497-506.

Ansari-Lari, M., & Tanideh, N. (2009). Changes in sex hormones and offspring sex ratio following gasoline exposure in male rats. *Comparative Clinical Pathology, 18*, 43-45.

Astolfi, P., Cuccia, M., & Martinetti, M. (2001). Paternal HLA genotype and offspring sex ratio. *Human Biology, 73*, 315-319.

Balli, K. S., Patton, W. C., Jacobson, J. D., Corselli, J., King, A., & Chan, P. J. (2004). Sperm velocity in seminal plasma and the association with gender of offspring. *Archives of Andrology, 50*, 37-40.

Barth, A. D. (2007). Sperm accumulation in the ampullae and cauda epididymides of bulls. *Animal Reproduction Science, 102*, 238-246.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*, 1-48.

Beck, K. J., Herschel, S., Hungershöfer, R., & Schwinger, E. (1976). The effect of steroid hormones on motility and selective migration of X- and Y-bearing human spermatozoa. *Fertility and Sterility, 27*, 407-412.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society B: Methodological, 57*, 289-300.

Briggs, M. (1973). Steroid hormones and the fertilizing capacity of spermatozoa. *Steroids, 22*, 547-553.

Burley, N. (1981). Sex ratio manipulation and selection for attractiveness. *Science, 211*, 721-722.

Cameron, E. Z. (2004). Facultative adjustment of mammalian sex ratios in support of the Trivers–Willard hypothesis: evidence for a mechanism. *Proceedings of the Royal Society B: Biological Sciences, 271*, 1723-1728.

Cameron, E. Z., Edwards, A. M., & Parsley, L. M. (2017). Developmental sexual dimorphism and the evolution of mechanisms for adjustment of sex ratios in mammals. *Annals of the New York Academy of Sciences, 1389*, 147-163.

Carter, J., Tinggi, U., Yang, X., & Fry, B. (2015). Stable isotope and trace metal compositions of Australian prawns as a guide to authenticity and wholesomeness. *Food Chemistry, 170*, 241-248.

Chandler, J. E., Canal, A. M., Paul, J. B., & Moser, E. B. (2002). Collection frequency affects percent Y-chromosome-bearing sperm, sperm head area and quality of bovine ejaculates. *Theriogenology, 57*, 1327-1346.

Chandra, A. K., Sengupta, P., Goswami, H., & Sarkar, M. (2013). Effects of dietary magnesium on testicular histology, steroidogenesis, spermatogenesis and oxidative stress markers in adult rats. *Indian Journal of Experimental Biology, 51*, 37-47.

Chen, X., Zhu, H., Wu, C., Han, W., Hao, H., Zhao, X., Du, W., Qin, T., Liu, Y., & Wang, D. (2012). Identification of differentially expressed proteins between bull X and Y spermatozoa. *Journal of Proteomics, 77*, 59-67.

Clark, A. B. (1978). Sex ratio and local resource competition in a prosimian primate. *Science, 201*, 163-165.

Colagar, A. H., Marzony, E. T., & Chaichi, M. J. (2009). Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. *Nutrition Research, 29*, 82-88.

Darszon, A., Labarca, P., Nishigaki, T., & Espinosa, F. (1999). Ion channels in sperm physiology. *Physiological Reviews, 79*, 481-510.

De Canio, M., Soggiu, A., Piras, C., Bonizzi, L., Galli, A., Urbani, A., & Roncada, P. (2014). Differential protein profile in sexed bovine semen: shotgun proteomics investigation. *Molecular BioSystems, 10*, 1264-1271.

Douhard, M. (2018). The role of fathers in mammalian sex allocation. *Mammal Review, 48*, 67-74.

Edwards, A. M., & Cameron, E. Z. (2014). Forgotten fathers: paternal influences on mammalian sex allocation. *Trends in Ecology & Evolution, 29*, 158-164.

Edwards, A. M., & Cameron, E. Z. (2017). Cryptic male choice: experimental evidence of sperm sex ratio and seminal plasma adjustment in relation to coital rate. *Reproduction, Fertility and Development, 29*, 1401-1404.

Eghbali, M., Alavi, S. S., Asri, R. S., & Khadem, A. M. (2010). Calcium, magnesium and total antioxidant capacity (TAC) in seminal plasma of water buffalo (*Bubalus bubalis*) bulls and their relationships with semen characteristics. *Veterinary Research Forum, 1*, 12-20.

Eisenberg, M. L., Murthy, L., Hwang, K., Lamb, D. J., & Lipshultz, L. I. (2012). Sperm counts and sperm sex ratio in male infertility patients. *Asian Journal of Andrology, 14*, 683-686.

Esfahani, M. H. N., Deemeh, M. R., Tavalaee, M., Sekhavati, M. H., & Gourabi, H. (2016). Zeta sperm selection improves pregnancy rate and alters sex ratio in male factor infertility patients: a double-blind, randomized clinical trial. *International Journal of Fertility & Sterility, 10*, 253-260.

Espinosa, F., & Darszon, A. (1995). Mouse sperm membrane potential: changes induced by Ca2+. *FEBS letters, 372*, 119-125.

Fallah, A., Mohammad-Hasani, A., & Colagar, A. H. (2018). Zinc is an essential element for male fertility: a review of Zn roles in men's health, germination, sperm quality, and fertilization. *Journal of Reproduction & Infertility, 19*, 69-81.

Fawcett, T. W., Kuijper, B., Pen, I., & Weissing, F. J. (2006). Should attractive males have more sons? *Behavioral Ecology, 18*, 71-80.

Galvani, A., & Johnstone, R. (1998). Sperm allocation in an uncertain world. *Behavioral Ecology and Sociobiology, 44*, 161-168.

Garratt, M., Bathgate, R., de Graaf, S. P., & Brooks, R. C. (2013). Copper-zinc superoxide dismutase deficiency impairs sperm motility and *in vivo* fertility. *Reproduction, 146*, 297-304.

Gibb, Z., Morris, L. H. A., Maxwell, W. M. C., & Grupen, C. G. (2011). Use of a defined diluent increases the sex-sorting efficiency of stallion sperm. *Theriogenology, 75*, 610-619.

Grant, V. J., & Irwin, R. (2005). Follicular fluid steroid levels and subsequent sex of bovine embryos. *Journal of Experimental Zoology A: Comparative Experimental Biology, 303*, 1120-1125.

Gutierrez-Adan, A., Granados, J., Pintado, B., & De La Fuente, J. (2001). Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured *in vitro*. *Reproduction, Fertility and Development, 13*, 361-365.

Hamilton, W. D. (1967). Extraordinary sex ratios. *Science, 156*, 477-488.

Hammadeh, M. E., & Hamad, M. F. (2009). Reactive oxygen species and antioxidant in seminal plasma and their impact on male fertility. *International Journal of Fertility & Sterility, 3*, 87-110.

Hatamoto, L. K., Sobrinho, C. A. B., Nichi, M., Barnabe, V. H., Barnabe, R. C., & Cortada, C. N. M. (2006). Effects of dexamethasone treatment (to mimic stress) and Vitamin E oral supplementation on the spermiogram and on seminal plasma spontaneous lipid peroxidation and antioxidant enzyme activities in dogs. *Theriogenology, 66*, 1610-1614.

Hemachand, T., & Shaha, C. (2003). Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS letters, 538*, 14-18.

Hilsenrath, R. E., Swarup, M., Bischoff, F. Z., Buster, J. E., & Carson, S. A. (1997). Effect of sexual abstinence on the proportion of X-bearing sperm as assessed by multicolor fluorescent *in situ* hybridization. *Fertility and Sterility, 68*, 510-513.

Hong, C. Y., Chiang, B. N., & Turner, P. (1984). Calcium ion is the key regulator of human sperm function. *The Lancet, 324*, 1449-1451.

Huang, Y. L., Tseng, W. C., Cheng, S. Y., & Lin, T. H. (2000). Trace elements and lipid peroxidation in human seminal plasma. *Biological Trace Element Research, 76*, 207-215.

Ishijima, S. A., Okuno, M., & Mohri, H. (1991). Zeta potential of human X- and Y-bearing sperm. *International Journal of Andrology, 14*, 340-347.

Jacobsen, R., Bostofte, E., Engholm, G., Hansen, J., Skakkebæk, N. E., & Møller, H. (2000). Fertility and offspring sex ratio of men who develop testicular cancer: a record linkage study. *Human Reproduction, 15*, 1958-1961.

James, W. H. (2008). Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Journal of Endocrinology, 198*, 3-15.

Jeannerat, E., Janett, F., Sieme, H., Wedekind, C., & Burger, D. (2017). Quality of seminal plasmas varies with type of stimulus at ejaculation. *Scientific Reports, 7*, 44339.

Jeannerat, E., Marti, E., Berney, C., Janett, F., Bollwein, H., Sieme, H., Burger, D., & Wedekind, C. (2018). Stallion semen quality depends on major histocompatibility complex matching to teaser mare. *Molecular Ecology, 27*, 1025-1035.

Johannisson, R., Leuschner, E., Hüppe, M., Hinrichs, F., Al-Hasani, S., Diedrich, K., Schwinger, E., & Mennicke, K. (2001). Increased frequency of X-bearing sperm in males from an infertility clinic: analysis by two-color fluorescence *in situ* hybridization. *Cytogenetic and Genome Research, 98*, 240-244.

Joseph, P. N., Sharma, R. K., Agarwal, A., & Sirot, L. K. (2015). Men ejaculate larger volumes of semen, more motile sperm, and more quickly when exposed to images of novel women. *Evolutionary Psychological Science, 1*, 195-200.

Kareskoski, M., & Katila, T. (2008). Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity. *Animal Reproduction Science, 107*, 249-256.

Kaya, A., Aksoy, M., & Tekeli, T. (2002). Influence of ejaculation frequency on sperm characteristics, ionic composition and enzymatic activity of seminal plasma in rams. *Small Ruminant Research, 44*, 153-158.

Kilgallon, S. J., & Simmons, L. W. (2005). Image content influences men's semen quality. *Biology Letters, 1*, 253-255.

Kimura, K., Spate, L., Green, M., & Roberts, R. M. (2005). Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. *Molecular Reproduction and Development, 72*, 201-207.

Kocer, A., Henry-Berger, J., Noblanc, A., Champroux, A., Pogorelcnik, R., Guiton, R., Janny, L., Pons-Rejraji, H., Saez, F., & Johnson, G. D. (2015). Oxidative DNA damage in mouse sperm chromosomes: size matters. *Free Radical Biology and Medicine, 89*, 993-1002.

Koziorowska-Gilun, M., Koziorowski, M., Strzeżek, J., & Fraser, L. (2011). Seasonal changes in antioxidant defence systems in seminal plasma and fluids of the boar reproductive tract. *Reproductive Biology, 11*, 37-47.

Lavoie, M. D., Tedeschi, J. N., Garcia-Gonzalez, F., & Firman, R. C. (2019). Exposure to male-dominated environments during development influences sperm sex ratios at sexual maturity. *Evolution Letters, 3*, 392-402.

Li, C. J., Wang, D., & Zhou, X. (2016). Sperm proteome and reproductive technologies in mammals. *Animal Reproduction Science, 173*, 1-7.

Luboshitzky, R., Kaplan-Zverling, M., Shen-Orr, Z., Nave, R., & Herer, P. (2002). Seminal plasma androgen/oestrogen balance in infertile men. *International Journal of Andrology, 25*, 345-351.

Madrid-Bury, N., Fernández, R., Jiménez, A., Pérez-Garnelo, S., Moreira, P. N., Pintado, B., De La Fuente, J., & Gutiérrez-Adán, A. (2003). Effect of ejaculate, bull, and a double swim-up sperm processing method on sperm sex ratio. *Zygote, 11*, 229-235.

Mahfouz, R., Sharma, R., Sharma, D., Sabanegh, E., & Agarwal, A. (2009). Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma. *Fertility and Sterility, 91*, 805-811.

Malo, A. F., Martinez-Pastor, F., Garcia-Gonzalez, F., Garde, J., Ballou, J. D., & Lacy, R. C. (2017). A father effect explains sex-ratio bias. *Proceedings of the Royal Society B: Biological Sciences, 284*, 20171159.

Mangiafico, S. (2016). *Summary and Analysis of Extension Program Evaluation in R, Version 1.18. 1*. North Brunswick, USA: Rutgers Cooperative Extension.

Mannowetz, N., Wandernoth, P. M., & Wennemuth, G. (2012). Glucose is a pH-dependent motor for sperm beat frequency during early activation. *PLoS One, 7*, e41030.

Miki, K. (2007). Energy metabolism and sperm function. *Society of Reproduction and Fertility Supplement, 65*, 309-325.

Min, Y., Sun, T., Niu, Z., & Liu, F. (2016). Vitamin C and vitamin E supplementation alleviates oxidative stress induced by dexamethasone and improves fertility of breeder roosters. *Animal Reproduction Science, 171*, 1-6.

Mirnamniha, M., Faroughi, F., Tahmasbpour, E., Ebrahimi, P., & Harchegani, A. B. (2019). An overview on role of some trace elements in human reproductive health, sperm function and fertilization process. *Reviews on Environmental Health, 34*, 339-348.

Mora, A. R., Meniri, M., Gning, O., Glauser, G., Vallat, A., & Helfenstein, F. (2017). Antioxidant allocation modulates sperm quality across changing social environments. *PLoS One, 12*, e0176385.

Müller, G., Martino-Andrade, A., Santos, A., Reghelin, A., Garcia, D., Sant'Ana, G., Spercoski, K., Meyer, K., Torres, S., & Júnior, V. S. (2012). Testicular testosterone: estradiol ratio in domestic cats and its relationship to spermatogenesis and epididymal sperm morphology. *Theriogenology, 78*, 1224-1234.

Navara, K. J. (2010). Programming of offspring sex ratios by maternal stress in humans: assessment of physiological mechanisms using a comparative approach. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 180*, 785-796.

Omu, A., Al-Azemi, M., Kehinde, E., Anim, J., Oriowo, M., & Mathew, T. (2008). Indications of the mechanisms involved in improved sperm parameters by zinc therapy. *Medical Principles and Practice, 17*, 108-116.

Oyeyipo, I. P., van der Linde, M., & du Plessis, S. S. (2017). Environmental exposure of sperm sex-chromosomes: a gender selection technique. *Toxicological Research, 33*, 315-323.

Peeker, R., Abramsson, L., & Marklund, S. (1997). Superoxide dismutase isoenzymes in human seminal plasma and spermatozoa. *Molecular Human Reproduction, 3*, 1061-1066.

Penfold, L., Holt, C., Holt, W., Welch, G., Cran, D., & Johnson, L. (1998). Comparative motility of X and Y chromosome-bearing bovine sperm separated on the basis of DNA content by flow sorting. *Molecular Reproduction and Development: Incorporating Gamete Research, 50*, 323-327.

Poiani, A. (2006). Complexity of seminal plasma: a review. *Behavioral Ecology and Sociobiology, 60*, 289-310.

R Core Team (2017). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*. Vienna, Austria.

Rahman, M. S., & Pang, M.-G. (2019). New biological insights on X and Y chromosome-bearing spermatozoa. *Frontiers in Cell and Developmental Biology, 7*, 1-19.

Robbins, W. A., Wei, F., Elashoff, D. A., Wu, G., Xun, L., & Jia, J. (2008). Y: X sperm ratio in boron-exposed men. *Journal of Andrology, 29*, 115-121.

Roblero, L., Guadarrama, A., Lopez, T., & Zegers-Hochschild, F. (1996). Effect of copper ion on the motility, viability, acrosome reaction and fertilizing capacity of human spermatozoa *in vitro*. *Reproduction, Fertility and Development, 8*, 871-874.

Røed, K. H., Holand, Ø., Mysterud, A., Tverdal, A., Kumpula, J., & Nieminen, M. (2007). Male phenotypic quality influences offspring sex ratio in a polygynous ungulate. *Proceedings of the Royal Society B: Biological Sciences, 274*, 727-733.

Saragusty, J., Hermes, R., Hofer, H., Bouts, T., Göritz, F., & Hildebrandt, T. B. (2012). Male pygmy hippopotamus influence offspring sex ratio. *Nature Communications, 3*, 1-5.

Sarkar, S. (1984). Motility, expression of surface antigen, and X and Y human sperm separation in *in vitro* fertilization medium. *Fertility and Sterility, 42*, 899-905.

Sartorelli, E. M. P., Mazzucatto, L. F., & de Pina-Neto, J. M. (2001). Effect of paternal age on human sperm chromosomes. *Fertility and Sterility, 76*, 1119-1123.

Segabinazzi, L. G., Silva, L. F., Okada, C., Medrado, F., Papa, F., & Alvarenga, M. A. (2018). Plugged ampullae in a donkey stallion (*Equus asinus*). *Journal of Equine Veterinary Science, 63*, 24-26.

Sharma, R. K., Pasqualotto, F. F., Nelson, D. R., Thomas Jr., A. J., & Agarwal, A. (1999). The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Human Reproduction, 14*, 2801-2807.

Stegmayr, B., Berggren, P.-O., Ronquist, G., & Hellman, B. (1982). Calcium, magnesium, and zinc contents in organelles of prostatic origin in human seminal plasma. *Scandinavian Journal of Urology and Nephrology, 16*, 199-203.

Stone, B. A., Alex, A., Werlin, L. B., & Marrs, R. P. (2013). Age thresholds for changes in semen parameters in men. *Fertility and Sterility, 100*, 952-958.

Talevi, R., Barbato, V., Fiorentino, I., Braun, S., Longobardi, S., & Gualtieri, R. (2013). Protective effects of *in vitro* treatment with zinc, d-aspartate and coenzyme q10 on human sperm motility, lipid peroxidation and DNA fragmentation. *Reproductive Biology and Endocrinology, 11*, 1-10.

Tamburrino, L., Marchiani, S., Minetti, F., Forti, G., Muratori, M., & Baldi, E. (2014). The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. *Human Reproduction, 29*, 418-428.

Tiido, T., Rignell-Hydbom, A., Jönsson, B., Giwercman, Y. L., Rylander, L., Hagmar, L., & Giwercman, A. (2005). Exposure to persistent organochlorine pollutants associates with human sperm Y: X chromosome ratio. *Human Reproduction, 20*, 1903-1909.

Trivers, R. L., & Willard, D. E. (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science, 179*, 90-92.

Tvrdá, E., Lukáč, N., Schneidgenová, M., Lukáčová, J., Szabó, C., Goc, Z., Greń, A., & Massányi, P. (2013). Impact of seminal chemical elements on the oxidative balance in bovine seminal plasma and spermatozoa. *Journal of Veterinary Medicine, 2013*, 125096

Van Dyk, Q., Mahony, M., & Hodgen, G. (2001). Differential binding of X-and Y-chromosome-bearing human spermatozoa to zona pellucida *in vitro*. *Andrologia, 33*, 199-205.

Williams, A. C., & Ford, C. L. (2001). The role of glucose in supporting motility and capacitation in human spermatozoa. *Journal of Andrology, 22*, 680-695.

You, Y. A., Kwon, W. S., Saidur Rahman, M., Park, Y. J., Kim, Y. J., & Pang, M. G. (2017). Sex chromosome-dependent differential viability of human spermatozoa during prolonged incubation. *Human Reproduction, 32*, 1183-1191.

Zhang, Q., Bai, Q., Yuan, Y., Liu, P., & Qiao, J. (2010). Assessment of seminal estradiol and testosterone levels as predictors of human spermatogenesis. *Journal of Andrology, 31*, 215-220.

Chapter 5: Number of reproductively active mares and stallion age predict foal sex in a managed population of feral horses

Abstract

Mammalian sex ratio research has historically focused on how mothers adjust offspring sex ratios. More recently, fathers have also been found to influence offspring sex ratios through skewed sperm sex ratio within an ejaculate. Factors linked to variation in sperm sex ratios include increasing mating frequency and age. Thus, these factors might also influence offspring sex ratios. Male mating frequency is influenced by both male competitiveness and the number of reproductively active females in the local population. In species that breed in stable and persistent polygynous groups, such as feral horses (*Equus ferus caballus*), the majority of mating occurs within breeding groups ('bands') such that a band stallion's mating frequency varies with the number of reproductively active mares in the band. Recent research demonstrates that increasing the ejaculation frequency of domestic stallions can increase the proportion of ejaculated X-chromosome-bearing spermatozoa (CBS). Thus, when band stallions have more reproductively active mares to mate, the likelihood of daughters being conceived is predicted to increase. I tested this hypothesis, examining whether the number of reproductively active mares in a band during the time of a foal's conception predicted foal sex in a population of managed feral horses. I also tested for other factors that may influence foal sex, specifically parental age and maternal body condition. I found that a higher number of reproductively active mares in a band during the time of conception was linked to more daughters, a result consistent with mating frequency-induced skews in sperm

sex ratios. More daughters were also born to younger stallions, again implicating paternal physiological effects on sex ratios.

Introduction

When offspring fitness differs by sex according to aspects of the local breeding environment, sex allocation theory predicts that parents will adjust the sex ratio of their offspring to maximise their fitness returns (e.g. Charnov *et al.*, 1981; Clark, 1978; Hamilton, 1967; Trivers & Willard, 1973). There is ample empirical evidence demonstrating that mothers adaptively adjust offspring sex ratios according to aspects of their local environment, such as population density, resource availability, social stress, and their own body condition and age (for reviews, see Geoffory & Douhard, 2019; Hardy, 1997; Navara, 2018; West, 2009). For example, maternal social stress has been linked to a greater proportion of daughters in several species (Firman, 2020, Ideta et al., 2009; Pratt & Lisk, 1989; for reviews, see Geoffory & Douhard, 2019; Navara, 2018), which has been attributed to elevations in cortisol in response to elevated maternal social stress (Firman, 2020). Conversely, there has been relatively little investigation into the contribution of fathers to offspring sex ratio variation, even though the theoretical considerations of sex allocation theory also apply to fathers (see Gomendio *et al.*, 2006) and plausible mechanisms exist for males to adjust sex ratios (see Cameron *et al.*, 2017). For example, mammalian males, as the heterogametic sex, can influence offspring sex ratios at fertilisation through biased sperm sex ratios within an ejaculate (reviewed in Douhard, 2018; Edwards & Cameron, 2014).

In mammals, sperm sex ratios have been shown to vary considerably between individual males (reviewed in Edwards & Cameron, 2014). Inter-individual variation in sperm sex ratios have been linked to genetic factors (e.g. Malo *et al.*, 2017; Szyda *et al.*, 2000) or hormonal conditions, particularly testosterone, during sperm development (James, 2004, 2006). Sperm sex ratios may also vary within an individual male across time (e.g. Chandler *et al.*, 2002). For example, in humans, advanced paternal age is linked to higher proportions of X-chromosome-bearing-spermatozoa (CBS) within the ejaculate (Sartorelli *et al.*, 2001; Stone *et al.*, 2013), suggesting that sperm sex ratios vary across a male's lifetime. Recently, increased male mating frequency in domestic stallions has also been linked to intra-male variation in sperm sex ratios, specifically a higher proportion of ejaculated X-CBS (Chapter 3 & 4). Since skews in the sperm sex ratio of an ejaculate can influence subsequent offspring sex ratios (e.g. Chandler *et al.*, 2007; Saragusty *et al.*, 2012), changes to males mating frequency could then affect the proportion of daughters conceived.

Male mating frequency is influenced by both a male's ability to secure mating opportunities and the number of breeding females in the local population, i.e. the operational sex ratio (Andersson, 1994; Arnold & Duvall, 1994; Emlen & Oring, 1977; Kvarnemo & Ahnesjo, 1996). In species where males monopolise groups of females for the purpose of mating only (female defence polygyny; Emlen & Oring, 1977), a male's mating success is tied to the size of the female group he is able to monopolise (e.g. Alberts *et al.*, 2006; Breuer *et al.*, 2010; Coltman *et al.*, 1999; Kaseda & Khalil, 1996; Mysterud *et al.*, 2003; Pemberton *et al.*, 1992). Within such breeding groups, as the number of reproductively active females increases, the mating frequency of males also increases (e.g. Byers *et al.*, 1994; Henry *et al.*, 1991; McClintock *et*

al., 1982; Preston *et al.*, 2001). For species that live in stable and persistent social groups, including females who are not reproductively active, such as feral horses (*Equus ferus caballus*) (Linklater, 2000; Ransom & Kaczensky, 2016), the mating frequency of a social group's male will depend on the size of the group and vary seasonally according to the reproductive status of each female in the group.

Feral horse populations are structured into stable year-round breeding groups ('bands') and less stable groups of non-breeding stallions ('bachelors') (Berger, 1986; Goodwin, 2007; Ransom & Kaczensky, 2016). A band consists of one to several mares, their immature offspring, and one or more stallions who monopolise the mating of band mares (Linklater, 2000). The majority of mating within a band is conducted by the band stallion, or in multi-stallion bands, the most dominant band stallion, with extra-band mating being generally rare (Bowling & Touchberry, 1990; Gray *et al.*, 2012; Linklater & Cameron, 2000; Miller, 1981). Horses are seasonally polyoestrous and mating predominantly occurs during the five to seven days of oestrous (Aurich, 2011; Curry *et al.*, 2007; Satué & Gardón, 2013). During this time, the stallion and mare mate several times per day for consecutive days (Asa *et al.*, 1979; McDonnell, 2000; Steinbjornsson & Kristjansson, 1999; Tarouco *et al.*, 2018; Tyler, 1972). Mares will return to oestrous approximately every 21 days until they either become pregnant (lasting 11 months) or the breeding season ends (Aurich, 2011; Crowell-Davis, 2007; Curry *et al.*, 2007; Nie *et al.*, 2007). Oestrous is not typically synchronised in mares, however, several mares in a band can be simultaneously in oestrous (see Asa *et al.*, 1979; Heitor & Vicente, 2011; Tarouco *et al.*, 2018). Observations from sexually rested domestic stallions introduced into a group of mares indicate that stallions mate with multiple mares per day, with mating frequency per oestrous mare increasing when more than one mare is in oestrus (Asa *et al.*,

1979; Bristol, 1982; Ginther, 1983; Heitor *et al.*, 2006; Heitor & Vicente, 2011). Thus, a band stallion's mating frequency is likely higher when there are more reproductively active mares present in the band.

Variation in stallion mating frequency may influence foal sex ratios through effects on ejaculated sperm sex ratios. In my previous work, I showed that in a population of regularly breeding domestic stallions, increasing the ejaculation frequency from once every two days to seven mating events within three days increased the proportion of X-CBS in successive ejaculates; however in a population of sporadically breeding stallions, ejaculations frequency was not associated with sperm sex ratios (Chapter 3 & 4). Feral stallions and domestic stallions breeding under similar conditions to feral stallions are observed to mate mares up to four times per day for consecutive days during oestrous (McDonnell, 2000; Steinbjornsson & Kristjansson, 1999; Tarouco *et al.*, 2018), a frequency that is comparable to my previous work in domestic stallions. Thus, when a band stallion's mating frequency is likely higher, i.e. when there are more reproductively active mares to breed, ejaculates might then contain higher proportions of X-CBS, potentially increasing the likelihood of daughters being conceived.

I tested whether a higher number of reproductively active mares in a band increased the proportion of daughters sired by the band stallion in a managed population of feral horses in North Dakota, USA. Usually, the number of reproductively active mares in a band is a measure of both stallion mating frequency and band size (specifically, the total number of mares in the band), thus making it difficult to separate the effects of stallion mating frequency from other factors that influence band size, such as stallion fighting ability, climatic conditions, the number of local bachelor stallions, and absolute mare density (Berger, 1986;

Pacheco & Herrera, 1997; Stevens, 1990; Waran, 2001). However, this population was used in an experimental trial of the immunocontraceptive GonaCon-Equine™ as a fertility control strategy for feral mares, which suppresses cycling in contracepted mares (Baker *et al*., 2018). Thus, the mating frequency of band stallions with contracepted mares in the band was artificially lowered, independently of the other factors that influence how many mares are in a band (i.e. band size). Thus, the influence of stallion mating frequency can be uncoupled from factors that influence band size since stallions are not mating with the contracepted mares in their band but are still influenced by the other factors that influence band size. Using the demographic data collected by Baker *et al*. (2018), I tested whether the number of reproductively active mares in a band (not pregnant or contracepted) during the time of conception predicted foal sex. I also controlled for maternal body condition (Cameron & Linklater, 2000) and parental age (Santos *et al.*, 2015; but see Monard *et al.*, 1997). Based on my findings that an increase in mating frequency increased the proportion of X-CBS (Chapter 3 & 4), I predicted that band stallions will sire more daughters when there are more reproductively active mares in the band during the time of conception.

This research has important implications for feral horse management. Population growth rates are generally restricted not by the number of males but by the number of breeding females and thus, skews towards males could slow population growth rates, whereas skews towards females could increase population growth rates (Wedekind 2002). As elevated population growth rates are generally undesirable for most feral horse populations, understanding the factors that increase the number of daughters born can be useful for the management of these populations.

Methods

Data collection

Data were obtained from nine years of observations (2009 – 2017; Baker *et al.,* 2018) conducted in Theodore Roosevelt National Park, a 19,000 ha area of native vegetation located in southwestern North Dakota, USA. Within the park, a population of feral horses, estimated at 150 - 175 individuals in 2017 (Baker *et al.,* 2018), is confined and managed as a "historical demonstration herd". Demographic data on the feral horse population in Theodore Roosevelt National Park was collected by myself and others for the Baker *et al.* (2018) study from 2009 to 2017 as part of an experimental trial to assess the efficacy of an initial and booster vaccination of the immunocontraceptive GonaCon-Equine™ (National Wildlife Research Center, Fort Collins, CO, USA) as a fertility control strategy for feral mares. GonaCon-Equine™ is a GnRH vaccine that stimulates a persistent immune response against endogenous GnRH (Imboden *et al.,* 2006). Transient infertility is induced by the suppression of GnRH release and ultimately, ovulation and behavioural oestrous (Powers *et al.*, 2014). GonaCon-Equine™ was administered twice (in 2009 and 2013) to each of 29 mares (apart from four mares that died before receiving the booster dose in 2013). The impacts on fertility, health, and behaviour following the administration of the initial and booster immunocontraceptive vaccine were assessed (see Baker *et al.,* 2018). GonaCon-Equine™ treatment was moderately effective following the initial dose in 2009, while the booster dose in 2013 was 100 % effective at preventing foaling in the following season and between 80-94 % effective in the subsequent two seasons (Baker *et al.*, 2018). There was no evidence of adverse physiological or behavioural effects on mares or foals, nor any abnormal reproductive behaviours between treated mares and stallions (Baker *et al.*, 2018).

All horses in the park were individually identified by unique colouration. The age, reproductive history, and genealogy data for each animal in the park have been maintained since 1993 (Baker *et al.*, 2018). Detailed demographic data, including foaling dates and band compositions, were collected by trained field technicians and volunteers from 2009 to 2017. During the intensive sampling period (1 March to 1 August, which incorporates the peak foaling season), 95 % of mares were observed at least weekly and 100 % every two weeks. From 1 August to 31 October, observations were less intense and more opportunistic depending on available personnel, weather, and road conditions and averaged once per month. Observations were not carried out between November and February. The population consisted of between 14 and 16 bands plus several bachelor stallions. At the commencement of the study (2009), there were 16 bands; all but two of these bands contained at least one treated mare. The number of mares in a band was calculated as the modal number of adult mares with membership in a band during peak breeding (March to June). Mares were considered adults if they were three years old and had left their natal band (Feist & McCullough, 1975; Goodloe *et al.*, 2000; Linklater *et al.*, 2004; Scorolli & Cazorla, 2010). Each band had a single band stallion and ranged from one to eight adult mares; the mean number of mares in a band was 4.2. Besides juveniles leaving their natal bands, movement between bands was minimal, mostly occurring when a band stallion was ousted by another stallion (see Baker *et. al*., 2018). Some bands contained adult sons (over three-years-old) who had not dispersed from their natal band; these stallions were not considered to be band stallions.

Annual foaling proportions were estimated by the presence or absence of foals. Mares were not considered pregnant unless a foal was observed (thus under-representing pregnancy rates but preventing false pregnancy recordings). Estimated birth dates were determined by foal presence and activity, presence of umbilicus, and the time since the mare was last observed without a foal. There were 317 foals recorded during the nine-year study. Thirteen foals died before their sex was recorded and were therefore excluded from sex ratio analysis, resulting in 304 foals of verified sex. Of these, 85 foals were conceived by mothers who had received GonaCon-Equine™ treatment, either because they were pregnant already at the time of treatment, the treatment failed to induce a sufficient immune response, or the effects of the treatment had abated with time. Although maternal body condition was documented to ensure no adverse side effect of treatment, it was not recorded consistently throughout the nine years of study at a scale that reflects the change in body condition at conception that predicts foal sex (see Cameron & Linklater, 2007). Thus, the following maternal condition proxies were used: estimated conception day (Henneke *et al.*, 1984), whether conception occurred during postpartum oestrous (within 18 days after partition; Ginther, 1992; Nagy *et al.*, 2000; Nagy *et al.*, 1998), and presence of foal in the previous year (Cameron *et al.*, 2001; Heitor & Vicente, 2011; Turner & Kirkpatrick, 2002), as each of these relates to maternal body condition. Mares in better body condition tend to conceive earlier in the season (Henneke *et al.*, 1984) and are more likely to conceive during the postpartum oestrous (Ginther, 1992; Nagy *et al.*, 2000; Nagy *et al.*, 1998). Additionally, mares that do not raise foals tend to be in better body condition in the following breeding season (Cameron *et al.*, 2001; Heitor & Vicente, 2011; Turner & Kirkpatrick, 2002).

To provide a measure of stallion mating frequency during the conception of each foal, the number of reproductively active mares in each band for the week of each foal's conception was calculated. Conception dates were estimated by subtracted 340 days from the estimated birth date of the foal (Seal & Plotka, 1983). The week of estimated conception was considered since a stallion will mate with the mare throughout the week of oestrous (Aurich, 2011; Curry *et al.*, 2007). The reproductive status of a mare was determined by the presence of a foal in the following season or successful GonaCon-Equine™ treatment status. A mare was considered reproductively inactive if she was determined pregnant, which was estimated by backdating from her own foal's birth date or successfully treated with contraception, which was determined by the lack of a foal following GonaCon-Equine™ treatment. A mare was considered reproductively active if no foal was observed in the following season or if she had not yet conceived, as determined by backdating her own foal's birth date. Mares that were unsuccessfully contracepted, as indicated by the presence of a foal following GonaCon-Equine™ treatment, were also considered reproductively active until after the week of estimated conception of their foals.

Genetic data on the population of horses in the park were collected by the National Park Service to assist in making management decisions. Paternity was genetically determined for 208 of the 304 recorded foals by the Animal Genetics Laboratory (Texas A&M, College Station, TX, USA) from hair sample follicles collected during scheduled roundups. Paternity was genetically determined as described by Ovchinnikov *et al.* (2018). Samples were used to sequence the control region of mitochondrial DNA (mtDNA) and 12 autosomal short tandem repeat (STR) markers. The incomplete paternity record was primarily due to foals dying

before samples were obtained, failure to capture foals, or the reduced sampling effort in 2013 and 2017. Although band paternity was high in this population (92 %), all foals with unverified paternity were excluded to prevent confounding effects of extra-band paternity. To prevent potential bias introduced by the uneven paternity sampling, the nine foals with established paternity in the two years of low paternity sampling effort, 2013 and 2017, were excluded. The final dataset consisted of 198 foals from 26 stallions and 81 mares over nine years; all analyses were carried out on this subset only.

Statistical analyses

All analyses were run in R (R Core Team, 2017). A multimodal inference approach (Burnham & Anderson, 2002) was used to identify which combinations of *a priori* selected variables best predicted foal sex. Predictor variables included were: the number of reproductively active mares, stallion age, mare age, mare treatment (yes, no), and maternal condition as measured by conception day, foal in the previous year (yes, no), and postpartum conception (yes, no). These variables together made up the global model, from which all subset candidate models in the analysis were derived from. I also assessed the influence of the number of mares in the band as compared to the number of reproductively active mares in the band; the number of reproductively active mares in the band diverges from the total number of mares during the breeding season as mares become pregnant and thus, reproductively inactive. Since it is not known how maternal and paternal factors together influence foal sex ratios, I had no *a priori* prediction as to which combination of maternal and paternal variables would best predict foal sex. For this reason, a multimodal inference approach was chosen as it compares and ranks a set of competing hypotheses (i.e. models)

to identify the hypothesis that is closest to reality within the set of models (Burnham & Anderson, 2002).

Continuous variables were rescaled and centred at zero to facilitate model convergence in analyses and to allow for comparison of relative influence among predictor variables. All variables were fit into a global binomial generalized linear mixed model (GLMM) with the *glmer* function in the *lme4* package (Bates *et al.*, 2014). Collinearity was assessed by with variance inflation factor (VIF) using the *vif.mer* function in the *MuMin* package (Barton, 2016). Although reproductively active mares become less available with season progression, collinearity was weak (Pearson's correlation: r = - 0.27) and both were retained in the models. Stallion and mare identity and foal conception year were fitted as random effects to account for non-independence of foals born in the same year and to the same stallion and/or mare.

All subset models from the global model were ranked according to AIC_c differences (Δi; Burnham & Anderson, 2002) with the *dredge* function in the *MuMin* package (Barton, 2016). This method was chosen as I had no *a priori* prediction as to what combination of maternal and paternal factors would best predict foal sex; while predictors were selected based on conclusions from previous studies (Cameron *et al.*, 1999; Monard *et al.*, 1997; Santos *et al.*, 2015; Chapter 3 & 4), how these effects might interact to ultimately determine foal sex has not been tested. All models were considered competitive if Δ_i was < 2.0 (Burnham & Anderson, 2002). Relative support for each model derived from the global model was assessed using weights calculated from AIC_c scores (wi; Burnham & Anderson, 2002). The

model.avg function in the *MuMin* package (Barton, 2016) was used to calculate conditional estimates and importance for each predictor averaged across all models. The influence of the modal number of mares in a band in comparison to the number of reproductively active mares in a band was not examined in the same model as they are inherently correlated. Therefore, the relative explanatory powers of the total number versus the number of reproductively active mares were examined in separate binomial GLMMs and ranked according to their AIC_c values, as described above.

Results

All foal sex ratios are reported as a proportion based on the number of female foals/total foals. The mean foal sex ratio across the nine years of the study was 0.52. Yearly foal sex ratios fluctuated between 0.76 and 0.35. The number of reproductively active mares during the time of a foal's conception was the most important predictor of foal sex (Table 5.1c). The likelihood of a female foal increased as the number of reproductively active mares in a band during the time of conception (ranging from one to nine) increased (OR: 1.68; 95 % CI: 1.22 – 2.33; Pr(>|z|) = 0.002; Table 5.1a; Figure 5.1a). This effect of the number of reproductively active mares was not an artefact of the total number of mares in the band since the alternate model with the modal number of mares had a probability of 1 % (Table 5.1b).

Stallion age at the time of conception also predicted foal sex (Table 5.1a). The age of stallions at the time of foal conception ranged from 2 to 18 years old, but 70 % of foals were fathered

by stallions between 8 and 14 years old. Stallions sired more sons as they aged (OR: 0.77; 95 % CI: 0.57 – 1.03; Pr(>|z|) = 0.07; Figure 5.1c). Stallion age was not related to the size of the band (Pearson's correlation; r = 0.12, p-value = 0.10). Other than the variation attributed to stallion age, there was little variation in foal sex related to the stallion (GLMM: stallion variance = 0.04 ± 0.02).

Conception day positively predicted the number of female foals (Table 5.1a; Figure 5.1b). Most foals (80.5 %) were born between April and June, with conception between March and May in the previous year. The number of daughters increased with later conception dates within the breeding season (OR: 1.43; 95 % CI: 1.02 – 1.97; Pr(>|z|) = 0.03; Figure 5.1b). The other proxy measures of maternal body condition - foal in the previous year and postpartum conception - did not predict foal sex (Table 5.1). Mare treatment status did not affect foal sex (Table 5.1). There was also little effect of maternal age on foal sex (Table 5.1). The youngest mother in this study was a yearling when she conceived and the oldest, 23 years old, but 85 % of mothers were between two and twelve years old at the time of conception. Additionally, there was no variation in foal sex attributed to mare identify (GLMM: mare variance = 0); however, this may be because most mares only had one or two foals during the study.

Table 5.1. Model ranking to determine a) the combination of *a priori* selected variables that best predicted foal sex and b) the effect of operational sex ratios (measured as the no. of reproductively active mares) versus adult sex ratios (measured as modal no. of mares) on foal sex among 198 foals born to 26 stallions and 81 mares in a feral horse population. Relative importance was calculated from w*i* and coefficient estimates were derived from conditional model averaging for each predictor variable across all 128 models run (c). Ranking is only presented for models with $\Delta i < 2$.

Rank	Model	df	logLikelihood	AIC_c	Δi	w*i*
a)						
1	*~ no. of reproductively active mares + conception day + stallion age*	7	-129.3	273.2	0	0.12
2	*~ no. of reproductively active mares + conception day*	6	-130.8	274.1	0.87	0.08
3	*~ no. of reproductively active mares + conception day + stallion age + mare age*	8	-129.1	274.9	1.64	0.05
4	*~ no. of reproductively active mares + conception day + stallion age + postpartum conception*	8	-129.2	275.2	1.95	0.05
32	*~ 1*	4	-135.3	278.8	5.55	0.007
b)						
1	*~ no. of reproductively active mares + conception day + stallion age*	7	-129.3	273.2	0.00	0.98
2	*~ modal no. of mares + conception day + stallion age*	7	-133.7	282.0	8.76	0.01

c)

Variables	Importance	Coefficient estimate
no. of reproductively active mares	91 %	0.48 ± 0.19
conception day	75 %	0.35 ± 0.17
stallion age	58 %	-0.27 ± 0.16
postpartum conception (yes)	30 %	-0.24 ± 0.38
mare age	28 %	0.08 ± 0.17
foal in previous year (yes)	26 %	-0.08 ± 0.33
treatment (yes)	26 %	0.06 ± 0.36

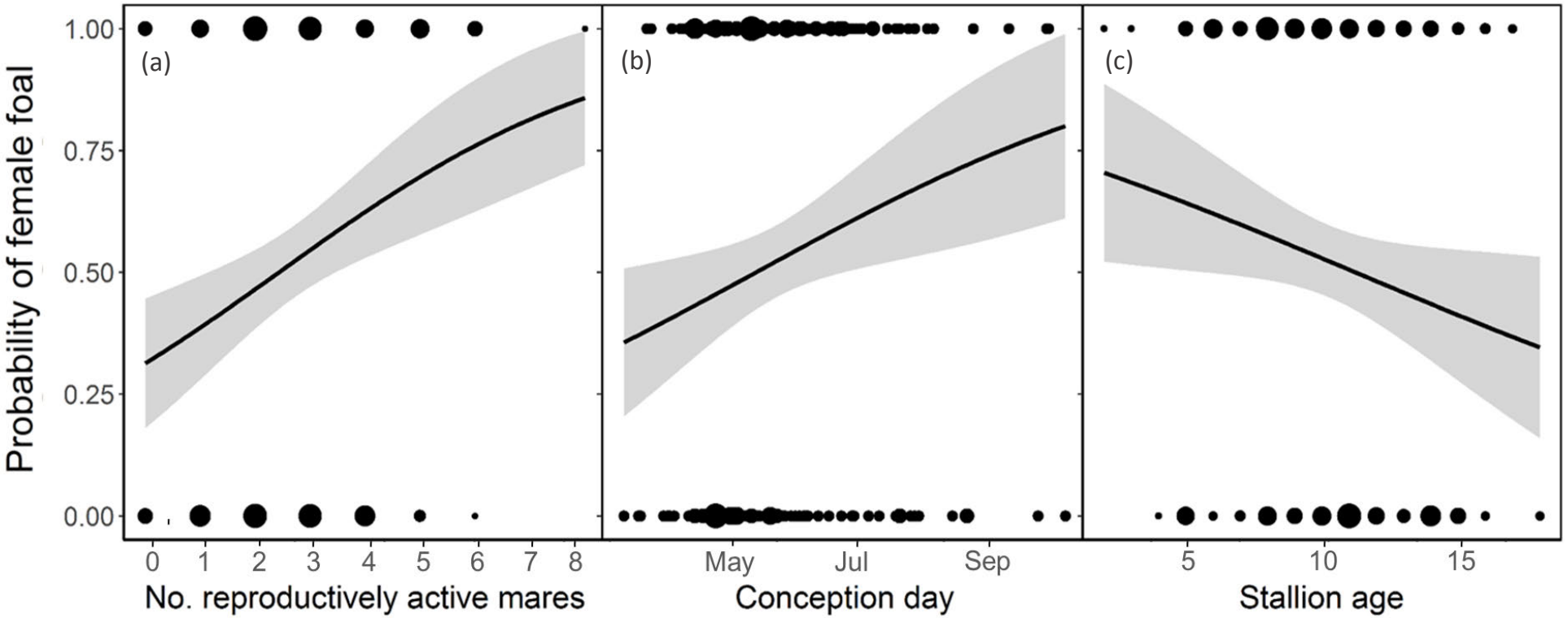

Figure 5.1. Predicted probabilities of the estimated number (no.) of reproductively active mares (a), estimated conception date (b), and stallion age (c) on foal sex (1 = female, 0 = male) among 198 foals born to 26 stallions and 81 mares in a feral horse population. For each variable, all other continuous variables in the full model were held constant at their means, respectively. The effect of the number of reproductively active mares and conception day were independent and contrasting since, as the breeding season progressed, there were fewer mares reproductively active.

Discussion

I tested whether the number of reproductively active mares in a band during the time of conception predicted the sex of the foal in a managed population of feral horses, while also testing the effects of parental ages and proxy measures of mare body condition. I had predicted that band stallions will sire more daughters when there are more reproductively active mares in the band during the time of conception based my findings in domestic stallions indicating an increase in the proportion of X-CBS when mating frequency increased (Chapter 3 & 4). Here I found results consistent with this hypothesis: more daughters followed more reproductively active mares in a band during the time of conception. This effect was not a function of the size of the band since the number of mares in the band did not predict foal sex, and thus could not be explained by mare density *per se*. The number of reproductively active mares diverges from the number of mares in a band as mares become pregnant and thus reproductively inactive. These results support the hypothesis that stallion mating frequency influences foal sex ratios. Although mating frequency and sperm sex ratios were not directly measured here, a stallion's mating frequency was likely higher when there were more reproductively active mares present (see Asa *et al.*, 1979; Bristol, 1982; Ginther, 1983; Heitor *et al.*, 2006; Heitor & Vicente, 2011). I have previously demonstrated that increasing the mating frequency of domestic breeding stallions to a similar frequency observed among feral stallions increased the proportion of ejaculated X-CBS (Chapter 3 & 4), shown elsewhere to influence the proportion of daughters born (e.g. Chandler *et al.*, 2007; Malo *et al.*, 2017; Saragusty *et al.*, 2012). Thus, the relationship between the number of reproductively active mares during conception and the proportion of daughters could have been mediated through mating frequency-induced skews in ejaculated sperm sex ratios.

An alternate explanation for my result is that the observed pattern was driven by maternal factors, most notably maternal social stress (Geffory & Douhard, 2019; Navara, 2018). Maternal social stress has been linked to biased offspring sex ratios in several mammalian species (Firman, 2020, Ideta et al., 2009; Linklater, 2007; Navara, 2010; Pratt & Lisk, 1989; Von Engelhardt *et al*., 2009). It is plausible that an increase in the number of reproductively active mares could increase social stress among those mares, since mares in oestrous will compete for access to the band stallion (Asa et al., 1979; Bristol, 1982; Ginther et al., 1983; Powell, 2008) and harass other mares in their band based on reproductive status (foal vs. no foal; Rutberg & Greenberg, 1990). When there are multiple mares in oestrous, social stress associated with competition and harassment might increase the number of daughters born (see Geffory & Douhard, 2019; Navara, 2018). Social stress in mares is linked to elevations in circulating cortisol (e.g. Alexander & Irvine, 1998; Nuñez et al., 2014), which has been linked to offspring sex ratio skews in other mammalian species (Ideta et al., 2009; Linklater, 2007; Navara, 2010; Pratt & Lisk, 1989; Pratt & Lisk, 1990).

A limitation in my study was that foal sex ratios were recorded among observed foals. Thus, pregnancy may have been underreported. According to Baker *et al.* (2018), determination of pregnancy by foal observation underestimated pregnancy by approximately 23 % in this population. This underestimation means that any sex-specific foal loss through abortion, stillbirth, or neonate death would not be accounted for here. Sex-specific neonate survival has been observed in other feral horse populations (Monard *et al.,* 1999). Thus, I cannot determine whether observed foal sex ratios reflect the sex ratios at the time of fertilization or are a result of sex-specific gestational or neonate loss (see Cameron *et al.*, 2017). However,

there was no evidence of sex-specific neonatal mortality amongst those foals that were recorded, suggesting that the link was indeed with more reproductively active mares in the band during conception being associated with more daughters in this population of feral horses.

Stallions sired fewer daughters as they aged, suggesting paternal physiological effects on sex ratios. Among the physiological hypotheses explaining paternal sex ratios is age-related variation in testosterone and the associated effects on spermatogenesis (James, 2008; Matsuo *et al.*, 2009; Sartorelli *et al.*, 2001; Stone *et al.*, 2013), with reduced paternal testosterone linked to a greater proportion of daughters in primates (Ansari-Lari & Tanideh, 2009; James, 2008; Perret, 2018). Testosterone tends to be lower in young and old males relative to prime-age males (Gray *et al.*, 1991; Johnson & Thompson Jr, 1983; Khalil *et al.*, 2009). Thus, both young and old fathers, compared to prime-age fathers, might have more daughters. Indeed, both in this population and elsewhere in other wild ungulates (Holand *et al.*, 2005; Sæther *et al.*, 2004; Vetter & Arnold, 2018), more daughters were born to young fathers. However, older stallions did not sire more daughters in this study. While studies in humans and domestic horses show more daughters are also born to old fathers (Garfinkel & Selvin, 1976; Matsuo *et al.*, 2009; Novitski & Sandler, 1956; Santos *et al.*, 2015), in the wild, male ungulates likely lose paternity due to declining fighting ability (Berger, 1986; Ransom & Kaczensky, 2016) before hypothesised age-related female-biasing effects can occur (e.g. Sartorelli *et al.*, 2001; Stone *et al.*, 2013). The oldest father in this study was 18 years old, but declines in testosterone and subsequent effects on spermatogenesis are generally not observed in domestic stallions until after the age of 20 (Blanchard *et al.*, 2012; Gottschalk *et*

al., 2016). However, it should be noted that the recorded effects of paternal aging on sex ratios in mammals have proven equivocal (Diefenbach *et al.*, 2019; Graffelman *et al.*, 1999; Lazarus, 2002; Martin *et al.*, 1995; Sæther *et al.*, 2004; Saragusty *et al.*, 2012) and the relationship between paternal aging, testosterone, and sex ratios remains to be empirically tested.

The relationship between paternal age and foal sex ratios could also be explained by a maternal response to perceived stallion attractiveness, in that older stallions might be considered more established and dominant, and thus more attractive mates by mares. Females are predicted to adjust their offspring sex ratios according to the attractiveness of the male (Booksmythe *et al*., 2017); however, evidence is equivocal as to whether younger or older males are perceived as more attractive in other ungulate species (see Sæther *et al*., 2004; Tanaka *et al*., 2018).

There was limited evidence that proxy measures of maternal body condition predicted foal sex. In the absence of a more direct measure of maternal body condition, known to be an important predictor of foal sex among feral mares (Cameron *et al.*, 1999), three proxy measures were used: estimated day of conception (Henneke *et al.*, 1984), foal in the previous year (Cameron *et al.*, 2001; Heitor & Vicente, 2011; Turner & Kirkpatrick, 2002), and conception during postpartum oestrous (Nagy *et al.*, 2000; Nagy *et al.*, 1998). Only the estimated day of conception predicted foal sex. More daughters were born as the breeding season progressed, an effect that was independent and contrasting to that of the number of reproductively active mares; more mares are reproductively active earlier in the breeding

season and more reproductively active mares are associated with more daughters. Since there is little evidence of a seasonal effect on foal sex ratios outside of the seasonal effects on maternal body condition (see Cameron & Linklater, 2007; Cameron *et al.*, 1999; Monard *et al.*, 1997), the higher proportion of sons born early in the season likely reflects the tendency of mares in better body condition to be able to conceive earlier in the season (Henneke *et al.*, 1984; Nagy *et al.*, 2000) and have more sons (Cameron & Linklater, 2000, 2007; Cameron *et al.*, 1999). Mare body condition is an important determinant of success and timing of conception but is also influenced by other factors, such as stress, age, season, climate, and individual variation (Aurich, 2011; Ginther, 1992; Guerin & Wang, 1994; Nagy *et al.*, 1998; Seal & Plotka, 1983). Thus, other factors may have influenced whether a mare raised a foal in the previous season and whether conception occurs during postpartum oestrous, potentially confounding these measures as proxies for maternal body condition at the time of conception.

GonaCon-Equine™ treatment did not affect foal sex ratios when treatment failed to induce contraception or once efficacy had waned, in agreement with other findings (Gray *et al.*, 2010). I considered treatment in the analysis because GonaCon-Equine™ suppresses GnRH and will alter subsequent circulating progesterone, testosterone, and oestradiol, even if complete infertility is not achieved (Dalin *et al.*, 2002). Progesterone, testosterone, and oestradiol are implicated in sex ratio variation (reviewed in Navara, 2018). It should be noted that due to the limitation in pregnancy assessment, unsuccessful treatment could have had undetected sex-specific effects on gestational loss not captured by my analyses.

Conclusions

My results make an important contribution to the management strategies of both feral and endangered populations of horses. Feral horse management strategies are controversial and expensive. For example, modelling suggests that at least 80 % of mares must be treated to bring population growth rates to zero (Hobbs & Hinds, 2018). My results suggest that GonaCon-Equine™ treatment might be more effective than initially thought by reducing the number of reproductively active mares. More sons born among non-treated mares would contribute to reducing population growth in the long term. Thus, consideration should be made to the indirect effects of treatment on the sex ratio of foals born among non-treated mothers when assessing the potential effectiveness of GonaCon-Equine™ treatment for feral horse populations.

References

Alberts, S. C., Buchan, J. C., & Altmann, J. (2006). Sexual selection in wild baboons: from mating opportunities to paternity success. *Animal Behaviour, 72*, 1177-1196.

Alexander, S., & Irvine, C. (1998). The effect of social stress on adrenal axis activity in horses: the importance of monitoring corticosteroid-binding globulin capacity. *Journal of Endocrinology, 157*, 425-432.

Andersson, M. B. (1994). *Sexual Selection*. Princeton, USA: Princeton University Press.

Ansari-Lari, M., & Tanideh, N. (2009). Changes in sex hormones and offspring sex ratio following gasoline exposure in male rats. *Comparative Clinical Pathology, 18*, 43-45.

Arnold, S. J., & Duvall, D. (1994). Animal mating systems: a synthesis based on selection theory. *The American Naturalist, 143*, 317-348.

Asa, C. S., Goldfoot, D. A., & Ginther, O. J. (1979). Socio-sexual behavior and ovulatory cycles of ponies. *Hormones and Behavior, 13*, 49-65.

Aurich, C. (2011). Reproductive cycles of horses. *Animal Reproduction Science, 124*, 220-228.

Baker, D. L., Powers, J. G., Ransom, J. I., McCann, B. E., Oehler, M. W., Bruemmer, J. E., Galloway, N. L., Eckery, D. C., & Nett, T. M. (2018). Reimmunization increases contraceptive effectiveness of gonadotropin-releasing hormone vaccine (GonaCon-Equine) in free-ranging horses (*Equus caballus*): limitations and side effects. *PLoS One, 13*, e0201570.

Barton, K. (2016). Package "MuMIn": Multi-Model Inference. R package, Version 1.15.6.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*, 1-48.

Berger, J. (1986). *Wild Horses of the Great Basin: Social Competition and Population Size*. Chicago, USA: University of Chicago Press.

Blanchard, T. L., Varner, D. D., Love, C. C., Brinsko, S. P., & Morehead, J. P. (2012). Management options for the aged breeding stallion with declining testicular function. *Journal of Equine Veterinary Science, 32*, 430-435.

Bowling, A. T., & Touchberry, R. W. (1990). Parentage of Great Basin feral horses. *Journal of Wildlife Management, 54*, 424-429.

Breuer, T., Robbins, A. M., Olejniczak, C., Parnell, R. J., Stokes, E. J., & Robbins, M. M. (2010). Variance in the male reproductive success of western gorillas: acquiring females is just the beginning. *Behavioral Ecology and Sociobiology, 64*, 515-528.

Bristol, F. (1982). Breeding behaviour of a stallion at pasture with 20 mares in synchronized oestrus. *Journal of Reproduction and Fertility Supplement, 32*, 71-77.

Burnham, K. P., & Anderson, D. R. (2002). *Model Selection and Multimodel Inference* (2 ed.). New York, USA: Springer.

Byers, J. A., Moodie, J. D., & Hall, N. (1994). Pronghorn females choose vigorous mates. *Animal Behaviour, 47*, 33-43.

Cameron, E. Z., Edwards, A. M., & Parsley, L. M. (2017). Developmental sexual dimorphism and the evolution of mechanisms for adjustment of sex ratios in mammals. *Annals of the New York Academy of Sciences, 1389*, 147-163.

Cameron, E. Z., & Linklater, W. L. (2000). Individual mares bias investment in sons and daughters in relation to their condition. *Animal Behaviour, 60*, 359-367.

Cameron, E. Z., & Linklater, W. L. (2007). Extreme sex ratio variation in relation to change in condition around conception. *Biology Letters, 3*, 395-397.

Cameron, E. Z., Linklater, W. L., Minot, E., & Stafford, K. (2001). *Population dynamics 1994-98, and management, of Kaimanawa wild horses*. Wellington, NZ. Department of Conservation

Cameron, E. Z., Linklater, W. L., Stafford, K. J., & Veltman, C. J. (1999). Birth sex ratios relate to mare condition at conception in Kaimanawa horses. *Behavioral Ecology, 10*, 472-475.

Chandler, J. E., Canal, A. M., Paul, J. B., & Moser, E. B. (2002). Collection frequency affects percent Y-chromosome-bearing sperm, sperm head area and quality of bovine ejaculates. *Theriogenology, 57*, 1327-1346.

Chandler, J. E., Taylor, T. M., Canal, A. L., Cooper, R. K., Moser, E. B., McCormick, M. E., Willard, S. T., Rycroft, H. E., & Gilbert, G. R. (2007). Calving sex ratio as related to the predicted Y-chromosome-bearing spermatozoa ratio in bull ejaculates. *Theriogenology, 67*, 563-571.

Charnov, E. L., Los-den Hartogh, R., Jones, W., & van den Assem, J. (1981). Sex ratio evolution in a variable environment. *Nature, 289*, 27-32.

Clark, A. B. (1978). Sex ratio and local resource competition in a prosimian primate. *Science, 201*, 163-165.

Coltman, D. W., Bancroft, D. R., Robertson, A., Smith, J. A., Clutton-Brock, T. H., & Pemberton, J. M. (1999). Male reproductive success in a promiscuous mammal: behavioural estimates compared with genetic paternity. *Molecular Ecology, 8*, 1199-1209.

Crowell-Davis, S. L. (2007). Sexual behavior of mares. *Hormones and Behavior, 52*, 12-17.

Curry, M. R., Eady, P. E., & Mills, D. S. (2007). Reflections on mare behavior: social and sexual perspectives. *Journal of Veterinary Behavior, 2*, 149-157.

Dalin, A. M., Andresen, Ø., & Malmgren, L. (2002). Immunization against GnRH in mature mares: antibody titres, ovarian function, hormonal levels and oestrous behaviour. *Journal of Veterinary Medicine A, 49*, 125-131.

Diefenbach, D. R., Alt, G. L., Wallingford, B. D., Rosenberry, C. S., & Long, E. S. (2019). Effect of male age structure on reproduction in white-tailed deer. *Journal of Wildlife Management, 83*, 1368-1376.

Douhard, M. (2018). The role of fathers in mammalian sex allocation. *Mammal Review, 48*, 67-74.

Edwards, A. M., & Cameron, E. Z. (2014). Forgotten fathers: paternal influences on mammalian sex allocation. *Trends in Ecology & Evolution, 29*, 158-164.

Emlen, S. T., & Oring, L. W. (1977). Ecology, sexual selection, and the evolution of mating systems. *Science, 197*, 215-223.

Feist, J. D., & McCullough, D. R. (1975). Reproduction in feral horses. *Journal of Reproduction and Fertility Supplement, 23*, 13-18.

Firman, R. C. (2020). Exposure to high male density causes maternal stress and female-biased sex ratios in a mammal. *Proceedings of the Royal Society B*, 287, 20192909.

Garfinkel, J., & Selvin, S. (1976). A multivariate analysis of the relationship between parental age and birth order and the human secondary sex ratio. *Journal of Biosocial Science, 8*, 113-121.

Geffroy, B., & Douhard, M. (2019). The adaptive sex in stressful environments. *Trends in Ecology & Evolution*, 34, 628-640.

Ginther, O. J. (1983). Sexual behavior following introduction of a stallion into a group of mares. *Theriogenology, 19*, 877-886.

Ginther, O. J. (1992). *Reproductive Biology of the Mare: Basic and Applied Aspects* (2 ed.). Wisconsion, USA: Equiservices Cross Plains.

Ginther, O. J., Scraba, S. T., & Nuti, L. C. (1983). Pregnancy rates and sexual behavior under pasture breeding conditions in mares. *Theriogenology, 20*, 333-345.

Gomendio, M., Malo, A. F., Soler, A. J., Fernández-Santos, M. R., Esteso, M. C., García, A. J., Roldan, E. R., & Garde, J. (2006). Male fertility and sex ratio at birth in red deer. *Science, 314*, 1445-1447.

Goodloe, R. B., Warren, R. J., Osborn, D. A., & Hall, C. (2000). Population characteristics of feral horses on Cumberland Island, Georgia and their management implications. *Journal of Wildlife Management, 64*, 114-121.

Goodwin, D. (2007). Horse behaviour: evolution, domestication and feralisation. In N. Waran (Ed.), *The Welfare of Horses* (pp. 1-18). Berlin, Germany: Springer, Dordrecht.

Gottschalk, M., Sieme, H., Martinsson, G., & Distl, O. (2016). Analysis of breed effects on semen traits in light horse, warmblood, and draught horse breeds. *Theriogenology, 85*, 1375-1381.

Graffelman, J., Fugger, E., Keyvanfar, K., & Schulman, J. (1999). Human live birth and sperm–sex ratios compared. *Human Reproduction, 14*, 2917-2920.

Grant, V. J. (2007). Could maternal testosterone levels govern mammalian sex ratio deviations? *Journal of Theoretical Biology, 246*, 708-719.

Gray, A., Berlin, J. A., McKinlay, J. B., & Longcope, C. (1991). An examination of research design effects on the association of testosterone and male aging: results of a meta-analysis. *Journal of Clinical Epidemiology, 44*, 671-684.

Gray, M. E., Thain, D. S., Cameron, E. Z., & Miller, L. A. (2010). Multi-year fertility reduction in free-roaming feral horses with single-injection immunocontraceptive formulations. *Wildlife Research, 37*, 475-481.

Guerin, M., & Wang, X. (1994). Environmental temperature has an influence on timing of the first ovulation of seasonal estrus in the mare. *Theriogenology, 42*, 1053-1060.

Hamilton, W. D. (1967). Extraordinary sex ratios. *Science, 156*, 477-488.

Hardy, I. C. (1997). Possible factors influencing vertebrate sex ratios: an introductory overview. *Applied Animal Behaviour Science, 51*, 217-241.

Heitor, F., do Mar Oom, M., & Vicente, L. (2006). Social relationships in a herd of Sorraia horses: Part II. Factors affecting affiliative relationships and sexual behaviours. *Behavioural Processes, 73*, 231-239.

Heitor, F. P. B. D., & Vicente, L. A. M. (2011). Stallion mate choice and mare sexual behaviour in a herd of Sorraia horses (*Equus caballus*). *ISRN Zoology, 2011*, 705790.

Henneke, D., Potter, G., & Kreider, J. (1984). Body condition during pregnancy and lactation and reproductive efficiency of mares. *Theriogenology, 21*, 897-909.

Henry, M., McDonnell, S. M., Lodi, L. D., & Gastalt, E. L. (1991). Pasture mating behaviour of donkeys (*Equus minus*) at natural and induced oestrus. *Journal of Reproduction and Fertility, 44*, 77-86.

Hobbs, R. J., & Hinds, L. A. (2018). Could current fertility control methods be effective for landscape-scale management of populations of wild horses (*Equus caballus*) in Australia? *Wildlife Research*, *45*, 195-207.

Holand, Ø., Mysterud, A., Røed, K. H., Coulson, T., Gjøstein, H., Weladji, R. B., & Nieminen, M. (2005). Adaptive adjustment of offspring sex ratio and maternal reproductive effort in an iteroparous mammal. *Proceedings of the Royal Society B: Biological Sciences, 273*, 293-299.

Ideta, A., Hayama, K., Kawashima, C., Urakawa, M., Miyamoto, A., & Aoyagi, Y. (2009). Subjecting holstein heifers to stress during the follicular phase following superovulatory treatment may increase the female sex ratio of embryos. *Journal of Reproduction and Development, 55*, 529-533.

Imboden, I., Janett, F., Burger, D., Crowe, M. A., Hässig, M., & Thun, R. (2006). Influence of immunization against GnRH on reproductive cyclicity and estrous behavior in the mare. *Theriogenology*, *66*, 1866-1875.

James, W. H. (2004). Further evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Human Reproduction, 19*, 1250-1256.

James, W. H. (2006). Offspring sex ratios at birth as markers of paternal endocrine disruption. *Environmental Research, 100*, 77-85.

James, W. H. (2008). Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Journal of Endocrinology, 198*, 3-15.

Johnson, L., & Thompson Jr, D. L. (1983). Age-related and seasonal variation in the sertoli cell population, daily sperm production and serum concentrations of follicle-stimulating hormone, luteinizing hormone and testosterone in stallions. *Biology of Reproduction, 29*, 777-789.

Kaseda, Y., & Khalil, A. M. (1996). Harem size and reproductive success of stallions in Misaki feral horses. *Applied Animal Behaviour Science, 47*, 163-173.

Khalil, A. M., Nakahara, K., Tokuriki, M., Kaseda, Y., & Murakami, N. (2009). Variation in fecal testosterone hormone concentration with season and harem size in Misaki feral horses. *Journal of Veterinary Medical Science, 71*, 1075-1078.

Kvarnemo, C., & Ahnesjo, I. (1996). The dynamics of operational sex ratios and competition for mates. *Trends in Ecology & Evolution, 11*, 404-408.

Lavoie, M. D., Tedeschi, J. N., Garcia-Gonzalez, F., & Firman, R. C. (2019). Exposure to male-dominated environments during development influences sperm sex ratios at sexual maturity. *Evolution Letters, 3*, 392-402.

Lazarus, J. (2002). Human sex ratios: adaptations and mechanisms, problems and prospects. In I. C. Hardy (Ed.), *Sex Ratios: Concepts and Research Methods* (pp. 287-311). Cambridge, UK: Cambridge University Press.

Linklater, W. L. (2007). Translocation reverses birth sex ratio bias depending on its timing during gestation: evidence for the action of two sex-allocation mechanisms. Reproduction, *Fertility and Development, 19*, 831-839.

Linklater, W. L. (2000). Adaptive explanation in socio-ecology: lessons from the Equidae. *Biological Reviews, 75*, 1-20.

Linklater, W. L., & Cameron, E. Z. (2000). Tests for cooperative behaviour between stallions. *Animal Behaviour, 60*, 731-743.

Linklater, W. L., Cameron, E. Z., Minot, E. O., & Stafford, K. J. (2004). Feral horse demography and population growth in the Kaimanawa Ranges, New Zealand. *Wildlife Research, 31*, 119-128.

Malo, A. F., Martinez-Pastor, F., Garcia-Gonzalez, F., Garde, J., Ballou, J. D., & Lacy, R. C. (2017). A father effect explains sex-ratio bias. *Proceedings of the Royal Society B: Biological Sciences, 284*, 20171159.

Martin, R. H., Spriggs, E., Ko, E., & Rademaker, A. W. (1995). The relationship between paternal age, sex ratios, and aneuploidy frequencies in human sperm, as assessed by multicolor FISH. *American Journal of Human Genetics, 57*, 1395-1399.

Matsuo, K., Ushioda, N., & Udoff, L. C. (2009). Parental aging synergistically decreases offspring sex ratio. *Journal of Obstetrics and Gynaecology Research, 35*, 164-168.

McClintock, M. K., Anisko, J. J., & Adler, N. T. (1982). Group mating among Norway rats. Part II. The social dynamics of copulation: competition, cooperation, and mate choice. *Animal Behaviour, 30*, 410-425.

McDonnell, S. M. (2000). Reproductive behavior of stallions and mares: comparison of free-running and domestic in-hand breeding. *Animal Reproduction Science, 60*, 211-219.

Miller, L., Fagerstone, K., Kemp, J., Killian, G., & Rhyan, J. (2008). *Immune mechanisms and characterization of injection site reactions involved in the multi-year contraceptive effect of the GonaCon™ vaccine.* Paper presented at the Proceedings of the Vertebrate Pest Conference, UC Davis, USA.

Miller, R. (1981). Male aggression, dominance and breeding behavior in Red Desert feral horses. *Zeitschrift für Tierpsychologie, 57*, 340-351.

Moehlman, P. D. (2005). Endangered wild equids. *Scientific American, 292*, 86-93.

Monard, A. M., Duncan, P., Fritz, H., & Feh, C. (1997). Variations in the birth sex ratio and neonatal mortality in a natural herd of horses. *Behavioral Ecology and Sociobiology, 41*, 243-249.

Mysterud, A., Holand, Ø., Røed, K. H., Gjøstein, H., Kumpula, J., & Nieminen, M. (2003). Effects of age, density and sex ratio on reproductive effort in male reindeer (*Rangifer tarandus*). *Journal of Zoology, 261*, 341-344.

Nagy, P., Guillaume, D., & Daels, P. (2000). Seasonality in mares. *Animal Reproduction Science, 60*, 245-262.

Nagy, P., Huszenicza, G., Juhasz, J., Kulcsar, M., Solti, L., Reiczigel, J., & Abavary, K. (1998). Factors influencing ovarian activity and sexual behavior of postpartum mares under farm conditions. *Theriogenology, 50*, 1109-1119.

Navara, K. J. (2010). Programming of offspring sex ratios by maternal stress in humans: assessment of physiological mechanisms using a comparative approach. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 180*, 785-796.

Navara, K. J. (2018). *Choosing Sexes: Mechanisms and Adaptive Patterns of Sex Allocation in Vertebrates*. New York, USA: Springer.

Nie, G., Sharp, D., Robinson, G., Cleaver, B., & Porter, M. (2007). Clinical aspects of seasonality in mares. In R. Youngquist & W. Threlfall (Eds.), *Current Therapy in Large Animal Theriogenology* (pp. 68-73). St. Louis, USA: Saunders Elsevier.

Novitski, E., & Sandler, L. (1956). The relationship between parental age, birth order and the secondary sex ratio in humans. *Annals of Human Genetics, 21*, 123-131.

Nuñez, C. M., Adelman, J. S., Smith, J., Gesquiere, L. R., & Rubenstein, D. I. (2014). Linking social environment and stress physiology in feral mares (*Equus caballus*): group transfers elevate fecal cortisol levels. *General and Comparative Endocrinology, 196*, 26-33.

Nuñez, C. M., Scorolli, A., Lagos, L., Berman, D., & Kane, A. (2016). Management of free-roaming horses. In J. Ransom & P. Kaczensky (Eds.), *Wild Equids. Ecology, Management, and Conservation* (pp. 133-148). Baltimore, USA: Johns Hopkins University Press.

Ovchinnikov, I. V., Dahms, T., Herauf, B., McCann, B., Juras, R., Castaneda, C., & Cothran, E. G. (2018). Genetic diversity and origin of the feral horses in Theodore Roosevelt National Park. *PLoS One, 13*, e0200795.

Pacheco, M. A., & Herrera, E. A. (1997). Social structure of feral horses in the llanos of Venezuela. *Journal of Mammalogy, 78*, 15-22.

Pemberton, J., Albon, S., Guinness, F., Clutton-Brock, T., & Dover, G. (1992). Behavioral estimates of male mating success tested by DNA fingerprinting in a polygynous mammal. *Behavioral Ecology, 3*, 66-75.

Perret, M. (2018). Revisiting the Trivers-Willard theory on birth sex ratio bias: role of paternal condition in a Malagasy primate. *PLoS One, 13*, e0209640.

Powell, D. M. (2008). Female–female competition or male mate choice? Patterns of courtship and breeding behavior among feral horses (*Equus caballus*) on Assateague Island. *Journal of Ethology, 26*, 137-144.

Powers, J. G., Monello, R. J., Wild, M. A., Spraker, T. R., Gionfriddo, J. P., Nett, T. M., & Baker, D. L. (2014). Effects of GonaCon immunocontraceptive vaccine in free-ranging female Rocky Mountain elk (*Cervus elaphus nelsoni*). *Wildlife Society Bulletin, 38*, 650-656.

Pratt, N. C., & Lisk, R. D. (1989). Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (*Mesocricetus auratus*). *Reproduction*, 87, 763-769.

Pratt, N. C., & Lisk, R. D. (1990). Dexamethasone can prevent stress-related litter deficits in the golden hamster. *Behavioral and Neural Biology, 54*, 1-12.

Preston, B. T., Stevenson, I. R., Pemberton, J. M., & Wilson, K. (2001). Dominant rams lose out by sperm depletion. *Nature, 409*, 681-682.

R Core Team (2017). R: a language and environment for statistical computing. *R Foundation for Statistical Computing*. Vienna, Austria.

Ransom, J. I., Cade, B. S., & Hobbs, N. T. (2010). Influences of immunocontraception on time budgets, social behavior, and body condition in feral horses. *Applied Animal Behaviour Science, 124*, 51-60.

Ransom, J. I., & Kaczensky, P. (2016). *Wild Equids: Ecology, Management, and Conservation*. Baltimore, USA: John Hopkins University Press.

Ransom, J. I., Powers, J. G., Garbe, H. M., Oehler Sr, M. W., Nett, T. M., & Baker, D. L. (2014). Behavior of feral horses in response to culling and GnRH immunocontraception. *Applied Animal Behaviour Science, 157*, 81-92.

Rutberg, A. T., & Greenberg, S. A. (1990). Dominance, aggression frequencies and modes of aggressive competition in feral pony mares. *Animal Behaviour, 40*, 322-331.

Sæther, B. E., Solberg, E. J., Heim, M., Stacy, J. E., Jakobsen, K. S., & Olstad, R. (2004). Offspring sex ratio in moose *Alces alces* in relation to paternal age: an experiment. *Wildlife Biology, 10*, 51-57.

Santos, M. M., Maia, L. L., Nobre, D. M., Neto, J. F. O., Garcia, T. R., Lage, M. C. G. R., de Melo, M. I. V., Viana, W. S., Palhares, M. S., & da Silva Filho, J. M. (2015). Sex ratio of equine offspring is affected by the ages of the mare and stallion. *Theriogenology, 84*, 1238-1245.

Saragusty, J., Hermes, R., Hofer, H., Bouts, T., Göritz, F., & Hildebrandt, T. B. (2012). Male pygmy hippopotamus influence offspring sex ratio. *Nature Communications, 3*, 1-5.

Sartorelli, E. M. P., Mazzucatto, L. F., & de Pina-Neto, J. M. (2001). Effect of paternal age on human sperm chromosomes. *Fertility and Sterility, 76*, 1119-1123.

Satué, K., & Gardón, J. (2013). A review of the estrous cycle and the neuroendocrine mechanisms in the mare. *Journal of Steroids and Hormonal Science, 4*, 1-8.

Scorolli, A. L., & Cazorla, A. C. L. (2010). Demography of feral horses (*Equus caballus*): a long-term study in Tornquist Park, Argentina. *Wildlife Research, 37*, 207-214.

Seal, U., & Plotka, E. (1983). Age-specific pregnancy rates in feral horses. *Journal of Wildlife Management, 47*, 422-429.

Steinbjornsson, B., & Kristjansson, H. (1999). Sexual behaviour and fertility in Icelandhorse herds. *Pferdeheilkunde, 15*, 481-490.

Stevens, E. F. (1990). Instability of harems of feral horses in relation to season and presence of subordinate stallions. *Behaviour, 112*, 149-161.

Stone, B. A., Alex, A., Werlin, L. B., & Marrs, R. P. (2013). Age thresholds for changes in semen parameters in men. *Fertility and Sterility, 100*, 952-958.

Szyda, J., Simianer, H., & Lien, S. (2000). Sex ratio distortion in bovine sperm correlates to recombination in the pseudoautosomal region. *Genetical Research, 75*, 53-59.

Tarouco, A., Freitas, C., Neves, A., Gregory, R., & Mattos, R. (2018). Sexual and social behaviors of pony stallions and mares. *Animal Reproduction, 6*, 535-535.

Trivers, R. L., & Willard, D. E. (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science, 179*, 90-92.

Turner, A., & Kirkpatrick, J. (2002). Effects of immunocontraception on population, longevity and body condition in wild mares (*Equus caballus*). *Reproduction Supplement, 60*, 187-195.

Tyler, S. J. (1972). The behaviour and social organization of the New Forest ponies. *Animal Behaviour Monographs, 5*, 87-196.

Vetter, S. G., & Arnold, W. (2018). Effects of population structure and density on calf sex ratio in red deer (*Cervus elaphus*) - implications for management. *European Journal of Wildlife Research, 64*, 1-12.

Von Engelhardt, N., Kaiser, S., Sachser, N., Kemme, K., Groothuis, T., & Wewers, D. (2009). An unstable social environment affects sex ratio in guinea pigs: an adaptive maternal effect? *Behaviour*, *146*, 1513-1529.

Waran, N. K. (2001). The social behaviour of horses. In L. Keeling & H. Gonyou (Eds.), *Social Behaviour in Farm Animals* (pp. 247-274). Wallingford, UK: CAB International.

West, S. (2009). *Sex Allocation*. Princeton, USA: Princeton University Press.

Zabek, M. (2015). *Understanding population dynamics of feral horses in the Tuan and Toolara State Forest for successful long-term population management.* (PhD). The University of Queensland, Australia.

Chapter 6: Mating frequency and age do not predict foal sex in thoroughbred stallions

Abstract

Variation in offspring sex ratios has often been explained as an adaptive parental strategy to maximise inclusive fitness according to differential fitness returns from sons and daughters. Empirical research shows offspring sex ratio skews relative to maternal traits that are often consistent with theoretical predictions. However, inconsistencies have been observed between theory and observation, particularly in mammals. Some of the inconsistencies may be explained by unaccounted variation in paternal contributions through variation in sperm sex ratios. There is increasing evidence that sperm sex ratios vary in response to a range of factors, such as high mating frequency and advanced paternal age, which has been shown to predict offspring sex ratios. Here I tested whether variation in male mating frequency and parental age explained variation in the foal sex ratios produced by thoroughbred stallions (*Equus ferus caballus*) in the New Zealand Thoroughbred Racing Studbook, while largely controlling for maternal effects. These stallions were mating at exceptionally high frequencies for equines throughout the breeding season and across their lifetimes. I analysed the sex of 46,486 foals sired by 125 stallions and 22,025 mares conceived at a range of high stallion mating frequencies and parental ages. I found no evidence that stallion mating frequency or parental age predicted foal sex, suggesting that, variation in mating frequency and age do not influence sex ratios for these high-mating stallions.

Introduction

Sex allocation theory, which predicts that individual parents adjust offspring sex ratios according to the relative fitness returns from sons and daughters, has usually been interpreted in terms of maternal fitness (e.g. Charnov, 1982; Hamilton, 1967; Trivers & Willard, 1973). Consequently, empirical research has focused on mothers, confirming links between offspring sex ratios and aspects of the local maternal environment, including maternal condition, age, and social rank, as well as local population density and resource availability (reviewed in West, 2009). In mammals, empirical research confirms maternal ability to adjust sex ratios in line with theoretical predictions (e.g. Cameron & Linklater, 2007; Clutton-Brock *et al.*, 1984; Kohlmann, 1999; Kruuk *et al.*, 1999; Moore *et al.*, 2015). However, inconsistencies between theory and observation have been recorded (see Brown & Silk, 2002; Hewison & Gaillard, 1999; Sheldon & West, 2004), suggesting factors other than those relating to the maternal environment also influence mammalian sex ratios, such as paternal factors (Edwards & Cameron, 2014). While fathers have been historically dismissed from mammalian sex allocation, the growing body of work linking paternal traits to offspring sex ratio skews demonstrates that fathers also influence sex allocation (reviewed in Douhard, 2018; Edwards & Cameron, 2014).

Fathers should also theoretically produce the sex that will maximise their inclusive fitness (see Gomendio *et al.*, 2006). Male fitness can be enhanced by an ability to obtain more mating opportunities and an ability to establish paternity at high mating frequencies (Andersson, 1994; Arnold & Duvall, 1994; Pemberton *et al.*, 1992). Thus, males mating at a high frequency are more likely to achieve higher fitness. For example, males that are more

attractive to females likely have more mating opportunities (e.g. Rhodes *et al.*, 2005). Additionally, larger testes typically support higher sperm production rates such that males with larger testes are expected to maintain fertility at a higher mating frequency and outcompete rival males in sperm competition (Neely *et al.*, 1982; Preston *et al.*, 2012; Preston *et al.*, 2003; Schinckel *et al.*, 1984). Thus, sons may enhance the inclusive fitness of 'high-mating' fathers if sons inherit the traits that make their fathers successful (i.e. sexy son hypothesis; Weatherhead & Robertson, 1979).

Mammalian fathers could influence offspring sex ratios by modifying the sperm sex ratios in their ejaculates (see Edwards & Cameron, 2014). Historically, it has been assumed that sperm sex ratios were constrained to parity by meiosis, but more recent evidence shows that sperm sex ratios are variable (e.g. Chandler *et al.*, 1998; Edwards *et al.*, 2016; Lavoie *et al.*, 2019; Saragusty *et al.*, 2012; Chapter 3 & 4). Variation in sperm sex ratios has been linked to factors such as mating frequency (Edwards & Cameron, 2017; Hilsenrath *et al.*, 1997; Chapter 3), age (Sartorelli *et al.*, 2001; Stone *et al.*, 2013), and fertility (Gomendio *et al.*, 2006; Johannisson *et al.*, 2001). Causal physiological mechanisms might include systematic loss of or damage to developing X- and Y-chromosome-bearing spermatozoa (CBS) that establish subsequent skews in the ejaculate (e.g. Robbins *et al.*, 2008; Szyda *et al.*, 2000; Tiido *et al.*, 2005). For example, the effects of aging on spermatogenesis (reviewed in Kidd *et al.*, 2001) are hypothesised to be more deleterious for developing Y-CBS, thereby establishing X-CBS biases in the ejaculates of old males (Matsuo *et al.*, 2009; Sartorelli *et al.*, 2001; Stone *et al.*, 2013). Sperm sex ratios also vary according to conditions at the time of mating (e.g. Firman *et al.*, 2020; Lavoie *et al.*, 2019), including variation in mating frequency (e.g. Chandler *et al.*, 2002;

Edwards & Cameron, 2017; Hilsenrath *et al.*, 1997; Chapter 3 & 4). However, this effect potentially varies with different measures of mating frequency, e.g. weekly versus seasonal (see Chapter 3). For example, in line with the hypothesis that high-mating males will have more sons and low-mating males will have more daughters (Edwards & Cameron, 2017; Gomendio *et al.,* 2006), domestic stallions (*Equus ferus caballus*) with low seasonal mating frequencies produced X-CBS-biased ejaculates (Chapter 3). However, high mating stallions did not have more Y-CBS, as would be predicted. Instead, when mating frequency was increased to induce sexual exhaustion in high-mating stallions, ejaculated sperm sex ratios shifted towards X-CBS (Chapter 3 & 4). Consistent with these latter findings, feral stallions produced more daughters when more mares were available for mating (likely increasing mating frequency) during the week of conception (Chapter 5). Thus, male mating frequency at different time scales may have differential effects on sex ratios.

I tested whether stallion mating frequency and parental age predicted the sex of foals produced by thoroughbred stallions in the New Zealand Thoroughbred Racing Studbook. As detailed records are kept for each stallion in this studbook, I was able to assess variation in foal sex ratios among the 46,486 foals sired by 125 stallions across their mating lifetime. I tested whether daily, weekly, seasonal, and lifetime stallion mating frequency predicted the sex of foals. The stallions in this dataset were mating at far higher frequencies than is normal for feral stallions (see Kaseda & Khalil, 1996; Linklater *et al.*, 2004) and non-commercially breeding domestic stallions (e.g. Steinbjornsson & Kristjansson, 1999), providing an opportunity to test the influence of extraordinarily high mating frequencies on foal sex ratios. Additionally, stallions in this dataset commenced mating at younger ages and continued to

mate until later ages than most feral counterparts, thus testing age-related changes in the foal sex ratio of individuals at the far ends of the aging spectrum. Maternal body condition, an important predictor of foal sex in feral horse populations (Cameron & Linklater, 2007; Cameron *et al.*, 1999), is consistently higher and less variable among commercially breeding domestic mares (Pagan *et al.*, 2006) than feral mares (Cameron & Linklater, 2007; Cameron *et al.*, 1999), thus reducing variation in foal sex ratios from condition-mediated sex allocation.

Methods

Data collection

Mating records were obtained from the New Zealand Thoroughbred Racing Studbook, a publicly accessible online database. New Zealand Thoroughbred Racing does not allow the use of artificial insemination technology; mares and stallions must either be bred "in-hand", which means the stallion and mare are handled and bred in a highly controlled environment (Scoggin, 2014) or allowed to more naturally breed at will ('pasture breeding'). Complete stallion mating records were collected for stallions with at least 40 recorded mating events and 25 sexed foals from at least two years of records. Mating records dated from 1989 to 2017 and all stallions were born between 1971 and 2011, to allow for stallions to have sex ratios recorded across their reproductive lifespan. Information obtained from the online database included the name and birth year of the stallion, the name and birth year of the mare, the date of each mating event, and the outcome of each mating event (i.e. if mating was successful and, if so, the sex of the foal). Data were checked for discrepancies in the

reported date of mating, and stallions with anomalous records were removed. This resulted in a final sample size of 46,486 foals from 125 stallions and 22,025 mares.

The following predictors were extracted for each of the 46,486 foals: the number of mating events occurring on the day of conception (*mate day*), the number of mating events occurring in the five days prior to conception (*5 days*), and the order in which conception fell within the season (*season order*). It was not possible to determine the exact sequence of a stallion's mating events within a single day as the sequence was not recorded in the studbook. Consequently, mating events occurring on the same day were randomly assigned an order within that day. Thus, the measures of *5 days* and *season order* reflect the order of mating events between days but not within days. These variables were selected based on previous findings that suggest sperm sex ratios vary in response to both short-term and season-long mating frequency (Chapter 3 & 4).

Statistical analysis

All statistical analyses were completed in R (R Core Team, 2017). The power to identify a 2% difference in foal sex ratios for the given sample size was calculated in G*Power (Erdfelder *et al.*, 1996) for binomial logistic regression. To assess whether lifetime foal sex ratios of individual stallions differed from parity, exact binomial tests were run with the *binom.test* function with a false discovery rate (FDR) adjustment for multiple tests (Benjamini & Hochberg, 1995).

A multimodal inference approach (Burnham & Anderson, 2002) was used to identify which combinations of a priori selected variables best predicted foal sex. The global binomial generalized mixed model (GLMM) was fit with *glmer* in the *lme4* package (Bates *et al.*, 2014) with the following fixed factors: *mate day, 5 days, season order, stallion age,* and *mare age*. All subset candidate models in the analysis were derived from this global model containing all variables of interest. To account for the potential that other factors influence paternal sex ratios, independent to mating frequency or age, stallion identity was specified as a random effect. Mare identify was also included as a random factor to account for individual variation in maternal sex ratios. Each factor varied in scale, making model convergence difficult; thus, each variable was rescaled and centred at zero. Collinearity was assessed by variance inflation factor (VIF) using the *vif.mer* function in the *MuMin* package (Barton, 2016). There was no evidence of collinearity, therefore, all selected variables were retained in the global model. To determine which combination of *a priori* selected factors best predicted foal sex, all subsets of the global model were ranked according to AIC differences (Δi; Burnham & Anderson, 2002) using the *dredge* function (MuMin package; Barton, 2016). This method was chosen because, although evidence suggests that stallion mating frequency influences foal sex ratios, it is not known how mating frequency at different time scales or different parental ages influence foal sex ratios. Thus, I had no *a priori* prediction as to which combination of these variables would best predict foal sex. Support for each model derived from the global model was assessed using weights derived from AIC (wi; Burnham & Anderson, 2002). Models were considered competitive if AIC fell within a delta of two (Burnham & Anderson, 2002).

Results

In total, 46,486 foals sired by 125 stallions and 22,025 mares were assessed. The number of foals sired per stallion ranged from 27 to 1,847 and averaged 366 ± 27.3 per stallion. The mean number of mating events in a stallion's lifetime was 559.26 ± 40.9, with the highest at 2,494 mating events. Descriptive statistics of each predictor included in analyses are provided in Table 6.1. The foal sex ratio (female/total) of all foals was 0.49, which did not deviate from parity (exact binomial test; $p = 0.24$). Despite the wide range of lifetime sex ratios ranging from 0.4 to 0.6 (Figure 6.1), no stallion had a statistically biased mean foal sex ratio.

In total, 32 models were compared. There was little support for any particular model and, with the null model ranking the third highest, little evidence that any of the selected variables explained variation in foal sex (Table 6.2) despite the sample size providing a power of 99.9 to detect up to 2% differences in foal sex ratios There was little variation in foal sex ratios attributed to either stallion (GLMM: stallion variance = 0.001 ± 0.03) or mare identity (GLMM: mare variance = 0.02 ± 0.15).

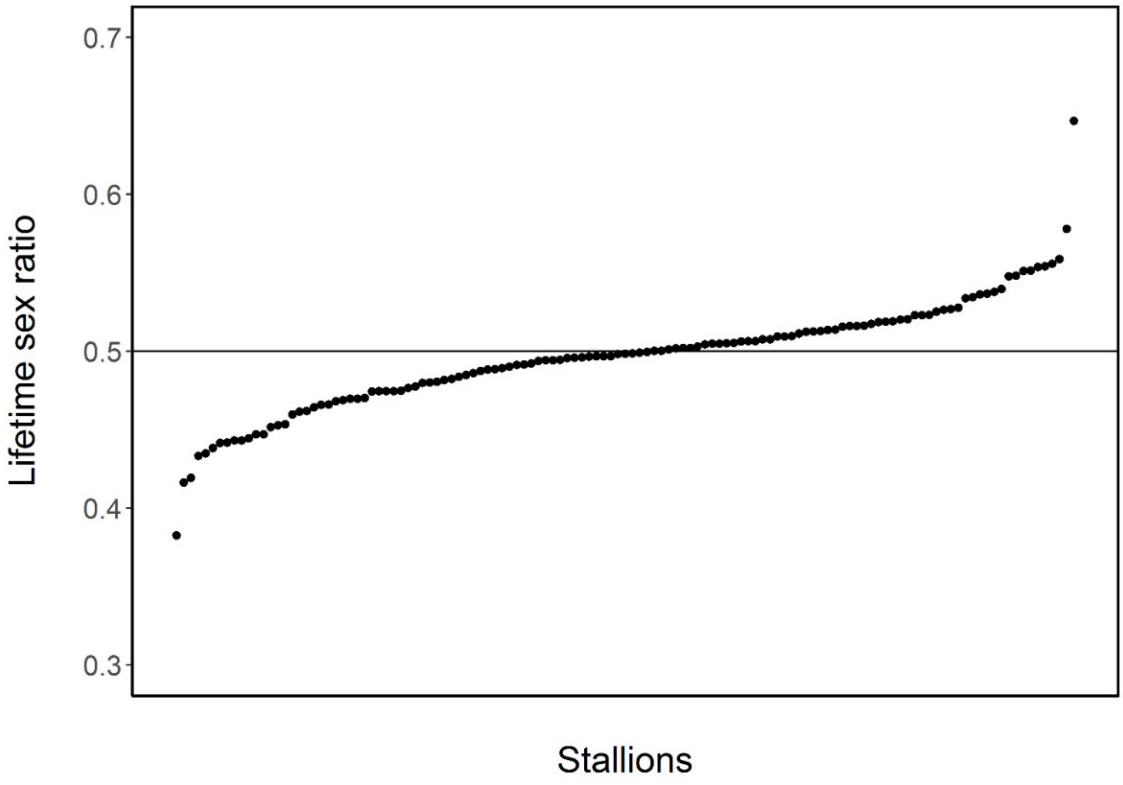

Figure 6.1. Lifetime foal sex ratios (female/total) of 125 thoroughbred stallions from the New Zealand Thoroughbred Racing Studbook.

Table 6.1. Summary of predictor variables included in the global model testing whether any measure of stallion mating rate or parental age predicted foal sex among 46,486 foals sired by 125 stallions and 22,025 mares.

Variable	Min	Max	Mean
season order	1	260	47.48
mate day	1	6	1.68
5 days	1	20	5.28
stallion age (years)	3	27	10.57
mare age (years)	2	26	10.29

Table 6.2. Summary of model selection to determine which combination of *a priori* selected measures of stallion mating rate and parental age best predicted foal sex among 46,486 foals sired by 125 stallions and 22,025 mares. Only models with delta less than two are presented.

Rank	Model	df	logLikelihood	AIC	Δ_i	w_i
1	~ *season order*	4	-32218	64443.9	0.00	0.08
2	~ *five days*	4	-32218	64444.1	0.19	0.07
3	~ *null*	3	-32219.2	64444.4	0.49	0.06
4	~ *five days + mare age*	5	-32217.3	64444.5	0.63	0.06
5	~ *mare age + season order*	5	-32217.3	64444.6	0.73	0.06
6	~ *mate day*	4	-32218.3	64444.7	0.76	0.06
7	~ *mare age*	4	-32218.4	64444.9	0.97	0.05
8	~ *mate day + season order*	5	-32217.5	64445.0	1.14	0.05
9	~ *mare age + mate day*	5	-32217.6	64445.1	1.22	0.04
10	~ *five days + season order*	5	-32217.6	64445.3	1.35	0.04
11	~ *mare age + mate day + season order*	6	-32216.9	64445.7	1.80	0.03
12	~ *five days + mate day*	5	-32217.9	64445.7	1.83	0.03
13	~ *five days + mare age + season order*	6	-32216.9	64445.9	1.97	0.03
14	~ *season order + stallion age*	5	-32217.9	64445.9	1.99	0.03

Discussion

I tested whether mating frequency and age predict the sex of foals sired by 125 stallions in the New Zealand Thoroughbred Racing Studbook. My sample size provided power of 99.9 to detect up to a 2 % difference in foal sex ratios, placing this amongst the largest investigations of paternal sex allocation in a non-human mammal. Although there was a wide range of lifetime foal sex ratios produced by individual stallions, my analyses did not find any stallion that produced statistically biased foal sex ratios. Moreover, neither stallion mating frequency nor age predicted foal sex. Since the analysis accounted for variation in sex ratios relating to paternal factors other than mating frequency (e.g. Lavoie *et al.*, 2019; Szyda *et al.*, 2000; Van Hooft *et al.*, 2010), the most parsimonious explanation for these results is that stallion mating frequency and age do not consistently alter sex ratios among these thoroughbreds.

The stallions in this dataset mated at higher daily, seasonal, and lifetime frequencies than is normal for feral stallions. Mating for feral stallions is primarily restricted to spring and to a limited number of mares (see Kaseda & Khalil, 1996; Linklater *et al.*, 2004), whereas these thoroughbred stallions mated with hundreds of mares per season and mating occurred both earlier and later in the season. Thus, over the course of their lifetimes, these stallions experienced extremely high mating frequencies for horses, but these high mating frequencies did not alter foal sex ratios. However, it should be noted that only the effects of very high mating frequencies were tested here. Thus, the effects of a low mating frequency on sex ratios were not assessed, which my previous results link to X-CBS biases (Chapter 3).

The results of this analysis are an interesting contrast to my previous work that demonstrated a shift in sex ratios with an increase in mating frequency among high-mating stallions (Chapter 3, 4, 5). This disparity in effects suggests that the effect of mating frequency on sex ratios is complex and may depend on other aspects of the breeding environment. Without data on sperm sex ratios both at ejaculation and at fertilization for each mating event, causal explanations for the mixed results cannot be determined. However, there were some differences between mating frequencies and other mating conditions of stallions in this and previous studies that are worth noting. Firstly, stallions in this study had little opportunity for sexual rest; between 1 August and 1 March, the median number of sexual rest days for stallions was one. Variation in the duration of sexual rest prior to ejaculation may contribute variation to sex ratios due to differential effects of epididymal sperm storage in the on X- and Y-CBS (Hendricks *et al.*, 2008; Hilsenrath *et al.*, 1997; You *et al.*, 2017). While my previous research showed that X- and Y-CBS do not have differential death rates in epididymal storage (Chapter 3), other studies suggest that there are more subtle sex-specific effects on stored spermatozoa (Hendricks *et al.*, 2008; Hilsenrath *et al.*, 1997; You *et al.*, 2017). The spermatozoa in the ejaculates of these stallions would have spent minimal time in storage and thus, would not have experienced the storage conditions that are hypothesised to contribute variation in sex ratios (see Hendricks *et al.*, 2008; You *et al.*, 2017).

Secondly, other mating factors, such as differences in mare familiarity, might influence the patterns of sperm allocation and depletion across multiple ejaculations that potentially facilitate shifts in sperm sex ratios. My previous results demonstrated that fewer spermatozoa were ejaculated across sequential mating events with familiar mares, a decline

that was greater for Y-CBS (Chapter 3 & 4). This shift could have related to variation in sperm allocation across multiple mating events with familiar mares. Female novelty predicts patterns of sperm allocation in that fewer spermatozoa are ejaculated per successive mating event with familiar females than with novel females (Alvarez-Fernandez *et al.*, 2019; Dewsbury, 1981; Joseph *et al.*, 2015; Pizzari *et al.*, 2003). In agreement, other studies demonstrating a relationship between mating frequency and sex ratios have also occurred over multiple mating events with the same group of females (Bartoš & Trojan, 1988; Edwards & Cameron, 2017). In contrast, the stallions in this study mated with a different and likely unfamiliar mare in each mating event, which could have altered patterns of sperm allocation across multiple mating events that potentially shift sperm sex ratios. However, while mare traits such as genetic compatibility and oestrous status have been shown to influence sperm allocation in horses (Burger *et al.*, 2017; Jeannerat *et al.*, 2017), the effects of mare novelty on sperm allocation have not yet been empirically demonstrated. Moreover, the influence of mare identity, including novelty, on sperm sex ratios remains to be tested.

Stallions in this study commenced mating earlier in life and continued mating later than is usual for feral stallions (Bowling & Touchberry, 1990; Feh, 1990; Kaseda & Khalil, 1996), thus providing an opportunity to test foal sex ratios at both ends of the paternal age spectrum. I found no evidence that stallion or mare age predicted variation in foal sex ratios. Previous findings are equivocal as to whether parental age influences sex ratios (see Arnold *et al.,* 1997; Graffelman *et al.*, 1999; Hewison *et al.*, 2002; Martin *et al.*, 1995; Matsuo *et al.*, 2009; Saltz, 2001; Santos *et al.*, 2015; Chapter 5). For example, while some studies in humans have documented an increase in the proportion of daughters sired by older men (Matsuo *et al.*,

2009; Jacobsen *et al.*, 1999), both Graffelman *et al.* (1999) and Martin *et al.* (1995) failed to find a corresponding shift in sperm sex ratios, suggesting that the shift related to factors other than age-related shifts in sperm sex ratios, such as the less frequent mating frequency of older men potentially delaying the timing of insemination relative to ovulation (James, 1975; Jacobsen *et al.,* 1999). Moreover, maternal age may influence offspring sex ratios only through age-related variation in maternal body condition (see Hewison *et al.*, 2002), which may be reduced among commercially breeding mares (see Pagan *et al.*, 2006; Scoggin, 2015). Given the sample size, this analysis provides strong evidence against either stallion or mare age influencing foal sex ratios.

Conclusions

Here I have presented evidence that stallion mating frequency and age do not predict the sex of the foal. The most parsimonious explanation is that mating frequency and age do not affect the sex ratios of thoroughbred stallions mating at extraordinarily high frequencies. However, this result is interesting when compared to my earlier findings that suggest a high mating frequency does influence sex ratios (Chapter 3, 4, 5). Thus, the influence of stallion mating frequency on sex ratios appears to be variable and contextual.

References

Alvarez-Fernandez, A., Borziak, K., McDonald, G. C., Dorus, S., & Pizzari, T. (2019). Female novelty and male status dynamically modulate ejaculate expenditure and seminal plasma proteome over successive matings in red junglefowl. *Scientific Reports, 9*, 5852.

Andersson, M. B. (1994). *Sexual Selection*. Princeton, USA: Princeton University Press.

Arnold, S. J., & Duvall, D. (1994). Animal mating systems: a synthesis based on selection theory. *The American Naturalist, 143*, 317-348.

Arnold, F., Rutstein, S., James, W. H., & Boklage, C. E. (1997). Sex ratio unaffected by parental age gap. *Nature*, *390*, 242-243.

Barton, K. (2016). Package "MuMIn": multi-model inference. R package, Version 1.15.6.

Bartoš, L., & Trojan, S. (1988). Effect of prolonged frequency of mating by the male rat on his fertility and on the sex ratio of his offspring. *Animal Reproduction Science, 17*, 271-279.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*, 1-48.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society B: Methodological, 57*, 289-300.

Blanchard, T. L., Varner, D. D., Love, C. C., Brinsko, S. P., & Morehead, J. P. (2012). Management options for the aged breeding stallion with declining testicular function. *Journal of Equine Veterinary Science, 32*, 430-435.

Bowling, A. T., & Touchberry, R. W. (1990). Parentage of Great Basin feral horses. *Journal of Wildlife Management, 54*, 424-429.

Brown, G. R., & Silk, J. B. (2002). Reconsidering the null hypothesis: is maternal rank associated with birth sex ratios in primate groups? *Proceedings of the National Academy of Sciences, 99*, 11252-11255.

Burger, D., Meuwly, C., Marti, E., Sieme, H., Oberthür, M., Janda, J., Meinecke-Tillmann, S., & Wedekind, C. (2017). MHC-correlated preferences in diestrous female horses (*Equus caballus*). *Theriogenology, 89*, 318-323.

Burnham, K. P., & Anderson, D. R. (2002). A practical information-theoretic approach. *Model Selection and Multimodel Inference.* New York, USA: Springer.

Cameron, E. Z., & Linklater, W. L. (2007). Extreme sex ratio variation in relation to change in condition around conception. *Biology Letters, 3*, 395-397.

Cameron, E. Z., Linklater, W. L., Stafford, K. J., & Veltman, C. J. (1999). Birth sex ratios relate to mare condition at conception in Kaimanawa horses. *Behavioral Ecology, 10*, 472-475.

Chandler, J. E., Canal, A. M., Paul, J. B., & Moser, E. B. (2002). Collection frequency affects percent Y-chromosome-bearing sperm, sperm head area and quality of bovine ejaculates. *Theriogenology, 57*, 1327-1346.

Chandler, J. E., Steinholt-Chenevert, H. C., Adkinson, R. W., & Moser, E. B. (1998). Sex ratio variation between ejaculates within sire evaluated by polymerase chain reaction, calving, and farrowing records. *Journal of Dairy Science, 81*, 1855-1867.

Clutton-Brock, T., Albon, S., & Guinness, F. (1984). Maternal dominance, breeding success and birth sex ratios in red deer. *Nature, 308*, 358-360.

Dewsbury, D. A. (1981). Effects of novelty of copulatory behavior: the Coolidge effect and related phenomena. *Psychological Bulletin, 89*, 464-482.

Douhard, M. (2018). The role of fathers in mammalian sex allocation. *Mammal Review, 48*, 67-74.

Edwards, A. M., & Cameron, E. Z. (2014). Forgotten fathers: paternal influences on mammalian sex allocation. *Trends in Ecology & Evolution, 29*, 158-164.

Edwards, A. M., & Cameron, E. Z. (2017). Cryptic male choice: experimental evidence of sperm sex ratio and seminal plasma adjustment in relation to coital rate. *Reproduction, Fertility and Development, 29*, 1401-1404.

Edwards, A. M., Cameron, E. Z., Pereira, J. C., & Ferguson-Smith, M. A. (2016). Paternal sex allocation: how variable is the sperm sex ratio? *Journal of Zoology, 299*, 37-41.

Erdfelder, E., Faul, F., & Buchner, A. (1996). GPOWER: a general power analysis program. *Behavior Research Methods, Instruments, & Computers, 28*, 1-11.

Feh, C. (1990). Long-term paternity data in relation to different aspects of rank for Camargue stallions, *Equus caballus*. *Animal Behaviour, 40*, 995-996.

Firman, R. C., Tedeschi, J. N., & Garcia-Gonzalez, F. (2020). Sperm sex ratio adjustment in a mammal: perceived male competition leads to elevated proportions of female-producing sperm. *Biology Letters, 16*, 20190929.

Gomendio, M., Malo, A. F., Soler, A. J., Fernández-Santos, M. R., Esteso, M. C., García, A. J., Roldan, E. R., & Garde, J. (2006). Male fertility and sex ratio at birth in red deer. *Science, 314*, 1445-1447.

Graffelman, J., Fugger, E. F., Keyvanfar, K., & Schulman, J. D. (1999). Human live birth and sperm-sex ratios compared. *Human Reproduction, 14*, 2917-2920.

Hendricks, K. E., Martins, L., & Hansen, P. J. (2008). Consequences for the bovine embryo of being derived from a spermatozoan subjected to post-ejaculatory aging and heat shock: development to the blastocyst stage and sex ratio. *Journal of Reproduction and Development, 55*, 69-74.

Hewison, A. M., & Gaillard, J. M. (1999). Successful sons or advantaged daughters? The Trivers–Willard model and sex-biased maternal investment in ungulates. *Trends in Ecology & Evolution, 14*, 229-234.

Hewison, A. M., Gaillard, J. M., Blanchard, P., & Festa-Bianchet, M. (2002). Maternal age is not a predominant determinant of progeny sex ratio variation in ungulates. *Oikos, 98*, 334-339.

Hilsenrath, R. E., Swarup, M., Bischoff, F. Z., Buster, J. E., & Carson, S. A. (1997). Effect of sexual abstinence on the proportion of X-bearing sperm as assessed by multicolor fluorescent *in situ* hybridization. *Fertility and Sterility, 68*, 510-513.

Jacobsen, R., Møller, H., & Mouritsen, A. (1999). Natural variation in the human sex ratio. *Human Reproduction*, *14*, 3120-3125.

James, W. H. (1975). Sex ratio and the sex composition of the existing sibs. *Annals of Human Genetics*, *38*, 371-378.

Jeannerat, E., Janett, F., Sieme, H., Wedekind, C., & Burger, D. (2017). Quality of seminal plasmas varies with type of stimulus at ejaculation. *Scientific Reports, 7*, 44339.

Johannisson, R., Leuschner, E., Hüppe, M., Hinrichs, F., Al-Hasani, S., Diedrich, K., Schwinger, E., & Mennicke, K. (2001). Increased frequency of X-bearing sperm in males from an infertility clinic: analysis by two-color fluorescence *in situ* hybridization. *Cytogenetic and Genome Research, 98*, 240-244.

Joseph, P. N., Sharma, R. K., Agarwal, A., & Sirot, L. K. (2015). Men ejaculate larger volumes of semen, more motile sperm, and more quickly when exposed to images of novel women. *Evolutionary Psychological Science, 1*, 195-200.

Kaseda, Y., & Khalil, A. M. (1996). Harem size and reproductive success of stallions in Misaki feral horses. *Applied Animal Behaviour Science, 47*, 163-173.

Kidd, S. A., Eskenazi, B., & Wyrobek, A. J. (2001). Effects of male age on semen quality and fertility: a review of the literature. *Fertility and Sterility, 75*, 237-248.

Kohlmann, S. G. (1999). Adaptive fetal sex allocation in elk: evidence and implications. *Journal of Wildlife Management, 63*, 1109-1117.

Kruuk, L. E., Clutton-Brock, T. H., Albon, S. D., Pemberton, J. M., & Guinness, F. E. (1999). Population density affects sex ratio variation in red deer. *Nature, 399*, 459-461.

Lavoie, M. D., Tedeschi, J. N., Garcia-Gonzalez, F., & Firman, R. C. (2019). Exposure to male-dominated environments during development influences sperm sex ratios at sexual maturity. *Evolution Letters, 3*, 392-402.

Linklater, W. L., Cameron, E. Z., Minot, E. O., & Stafford, K. J. (2004). Feral horse demography and population growth in the Kaimanawa Ranges, New Zealand. *Wildlife Research, 31*, 119-128.

Martin, R. H., Spriggs, E., Ko, E., & Rademaker, A. W. (1995). The relationship between paternal age, sex ratios, and aneuploidy frequencies in human sperm, as assessed by multicolor FISH. *American Journal of Human Genetics, 57*, 1395-1399.

Matsuo, K., Ushioda, N., & Udoff, L. C. (2009). Parental aging synergistically decreases offspring sex ratio. *Journal of Obstetrics and Gynaecology Research, 35*, 164-168.

Moore, E. P., Hayward, M., & Robert, K. A. (2015). High density, maternal condition, and stress are associated with male-biased sex allocation in a marsupial. *Journal of Mammalogy, 96*, 1203-1213.

Neely, J., Johnson, B., Dillard, E., & Robison, O. (1982). Genetic parameters for testes size and sperm number in Hereford bulls. *Journal of Animal Science, 55*, 1033-1040.

Pagan, J. D. (2009). *Advances in Equine Nutrition IV*. Versailles, Kentucky, USA: Nottingham University Press.

Pemberton, J., Albon, S., Guinness, F., Clutton-Brock, T., & Dover, G. (1992). Behavioral estimates of male mating success tested by DNA fingerprinting in a polygynous mammal. *Behavioral Ecology, 3*, 66-75.

Pizzari, T., Cornwallis, C. K., Løvlie, H., Jakobsson, S., & Birkhead, T. R. (2003). Sophisticated sperm allocation in male fowl. *Nature, 426*, 70-74.

Preston, B. T., Stevenson, I. R., Lincoln, G. A., Monfort, S. L., Pilkington, J. G., & Wilson, K. (2012). Testes size, testosterone production and reproductive behaviour in a natural mammalian mating system. *Journal of Animal Ecology, 81*, 296-305.

Preston, B. T., Stevenson, I. R., Pemberton, J. M., Coltman, D. W., & Wilson, K. (2003). Overt and covert competition in a promiscuous mammal: the importance of weaponry and testes size to male reproductive success. *Proceedings of the Royal Society B: Biological Sciences, 270*, 633-640.

R Core Team (2017). R: a language and environment for statistical computing. *R Foundation for Statistical Computing*. Vienna, Austria.

Rhodes, G., Simmons, L. W., & Peters, M. (2005). Attractiveness and sexual behavior: does attractiveness enhance mating success? *Evolution and Human Behavior, 26*, 186-201.

Robbins, W. A., Wei, F., Elashoff, D. A., Wu, G., Xun, L., & Jia, J. (2008). Y: X sperm ratio in boron-exposed men. *Journal of Andrology, 29*, 115-121.

Saltz, D. (2001). Progeny sex ratio variation in ungulates: maternal age meets environmental perturbation of demography. *Oikos, 94*, 377-384.

Santos, M. M., Maia, L. L., Nobre, D. M., Neto, J. F. O., Garcia, T. R., Lage, M. C. G. R., de Melo, M. I. V., Viana, W. S., Palhares, M. S., & da Silva Filho, J. M. (2015). Sex ratio of equine offspring is affected by the ages of the mare and stallion. *Theriogenology, 84*, 1238-1245.

Saragusty, J., Hermes, R., Hofer, H., Bouts, T., Göritz, F., & Hildebrandt, T. B. (2012). Male pygmy hippopotamus influence offspring sex ratio. *Nature Communications, 3*, 1-5.

Sartorelli, E. M. P., Mazzucatto, L. F., & de Pina-Neto, J. M. (2001). Effect of paternal age on human sperm chromosomes. *Fertility and Sterility, 76*, 1119-1123.

Schinckel, A., Johnson, R., & Kittok, R. (1984). Testicular development and endocrine characteristics of boars selected for either high or low testis size. *Journal of Animal Science, 58*, 675-685.

Scoggin, C. F. (2014). Natural service: in-hand breeding. In J. J. Dascanio & P. M. McCue (Eds.), *Equine Reproductive Procedures* (pp. 114-116): John Wiley & Sons.

Scoggin, C. F. (2015). Not just a number: effect of age on fertility, pregnancy and offspring vigour in thoroughbred brood-mares. *Reproduction, Fertility and Development, 27*, 872-879.

Sheldon, B. C., & West, S. A. (2004). Maternal dominance, maternal condition, and offspring sex ratio in ungulate mammals. *The American Naturalist, 163*, 40-54.

Steinbjornsson, B., & Kristjansson, H. (1999). Sexual behaviour and fertility in Icelandhorse herds. *Pferdeheilkunde, 15*, 481-490.

Stone, B. A., Alex, A., Werlin, L. B., & Marrs, R. P. (2013). Age thresholds for changes in semen parameters in men. *Fertility and Sterility, 100*, 952-958.

Szyda, J., Simianer, H., & Lien, S. (2000). Sex ratio distortion in bovine sperm correlates to recombination in the pseudoautosomal region. *Genetical Research, 75*, 53-59.

Tiido, T., Rignell-Hydbom, A., Jönsson, B., Giwercman, Y. L., Rylander, L., Hagmar, L., & Giwercman, A. (2005). Exposure to persistent organochlorine pollutants associates with human sperm Y: X chromosome ratio. *Human Reproduction, 20*, 1903-1909.

Van Hooft, P., Prins, H. H., Getz, W. M., Jolles, A. E., van Wieren, S. E., Greyling, B. J., van Helden, P. D., & Bastos, A. D. (2010). Rainfall-driven sex-ratio genes in African buffalo suggested by correlations between Y-chromosomal haplotype frequencies and foetal sex ratio. *BMC Evolutionary Biology, 10*, 1-11.

Weatherhead, P. J., & Robertson, R. J. (1979). Offspring quality and the polygyny threshold: "the sexy son hypothesis". *The American Naturalist, 113*, 201-208.

West, S. (2009). *Sex Allocation*. Princeton, USA: Princeton University Press.

You, Y. A., Kwon, W. S., Saidur Rahman, M., Park, Y. J., Kim, Y. J., & Pang, M. G. (2017). Sex chromosome-dependent differential viability of human spermatozoa during prolonged incubation. *Human Reproduction, 32*, 1183-1191.

General Discussion

In my thesis, I identified and addressed outstanding gaps in our current understanding of sex ratio adjustment in mammals. First, I aimed to test whether prenatal masculinisation or feminisation of female laboratory mice (*Mus musculus*) influenced their subsequent litter sex ratios. In doing so, my aim was to test if there are potential physiological constraints on the ability of some mothers to adjust sex ratios to the extent theory predicts. Second, I tested whether there was within-male variation in sperm sex ratios and seminal plasma that could link to paternal control over sex ratios. I measured sperm sex ratios and key components of the seminal plasma and their correlation to sperm sex ratios in multiple successive ejaculates of domestic stallions (*Equus ferus caballus*). I then assessed whether the intra-stallion variation I observed in sperm sex ratios corresponded to variation in foal sex ratios to test whether proximal modifiers of sperm sex ratios also influenced offspring sex ratios.

Prenatal effects on maternal sex ratios

As a litter-bearing species, female mice phenotypes are masculinised or feminised by intrauterine exposure to testosterone and oestradiol from endogenous, maternal, and placental sources as well as from neighbouring siblings *in utero* (Hu *et al*., 2015; Monclús & Blumstein, 2012; Vreeburg *et al*., 1983). In Chapter 2, I showed that phenotypic variation among females correlated with variation in litter sex ratios with more feminised females produced more daughters (see Figure 7.1). This work added to findings in other similar species, including mound-building mice (*Mus spicilegus*; Szenczi *et al.*, 2013), rabbits (*Oryctolagus cuniculus*; Bánszegi *et al.,* 2012), degus (*Octodon degus*; Correa *et al.*, 2016),

and grey mouse lemurs (*Microcebus murinus*; Perret, 2019), that showed that prenatal masculinisation and feminisation is an important source of variation in offspring sex ratios. Taken together, this suggests these effects may be widespread among litter-bearing eutherians.

I used the ratio of the second to fourth digit to assess the degree to which a female was prenatally masculinised or feminised. This approach allowed me to link the prenatal environment to later offspring sex ratio skews. Digit ratio is established by the relative exposure to testosterone and oestradiol during late prenatal development in rodents (Zheng & Cohn, 2011). Thus, my results specifically linked higher exposure to oestradiol relative to testosterone during late prenatal development to an increase in daughters. In contrast, most other studies linking the prenatal environment to offspring sex ratio skews have used anogenital distance (Bánszegi *et al.,* 2012; Clark et al., 1994; Correa *et al.*, 2016; Szenczi *et al.*, 2013). In contrast to digit ratio, rodent anogenital distance continues to vary with variation in testosterone and oestradiol after birth and into adulthood (Dušek *et al*., 2010; Kita *et al*., 2016; Mitchell, *et al*., 2015). Moreover, female rodents' anogenital distance can be masculinised by elevated testosterone as well as by elevated oestradiol (see Schwartz *et al*., 2018). Thus, assessing litter sex ratios against digit ratio provided a direct link to the prenatal effects that alter offspring sex ratio by specifically linking higher oestradiol to testosterone exposure during late prenatal developmental to female-biased litter sex ratios. Future experimental designs that increase a female's prenatal oestradiol relative to testosterone exposure during late gestation and then quantify subsequent physiological effects and litter sex ratios would further clarify the potential physiological mechanisms in rodents that link the prenatal environment to litter sex ratios in adulthood.

The proportion of the variation in litter sex ratios explained by prenatal feminisation was small (3 %) but statistically significant (Chapter 2). Similarly, in other species, prenatal effects also explain relatively small proportions of variation (e.g. 5.38 % in degus; Correa *et al*., 2016), particularly when compared to proximal maternal factors that are linked to sex ratio skews. For example, changes in maternal body mass explained 27 % of the variation in litter sex ratios in Richardson's ground squirrels (*Urocitellus richardsonii;* Ryan *et al.*, 2012) and changes in circulating blood glucose explained 54 % of the variation in litter sex ratios in laboratory mice (Cameron *et al.*, 2008). However, understanding sex allocation in mammals requires consideration of the many factors that influence sex ratios, including the subtle effects of the prenatal environment (Figure 7.1). My finding indicated that inclusion of digit ratio in tests of maternal sex allocation will account for some of these effects, thereby improving explanations of litter sex ratio variation. Digit ratio provides a measure of the relative exposure to testosterone and oestradiol *in utero* in a variety of mammalian species other than mice, including rats (Auger *et al*., 2013), lemurs (Nelson *et al*., 2010), and humans (Lutchmaya *et al.*, 2004; Manning, 2011; Ventura *et al.*, 2013; but see Hickey *et al.*, 2010; Hollier *et al.*, 2015).

My result could also have implications for sex allocation that extend beyond explaining the relatively small effect size in litter sex ratios if prenatal feminisation imposes subsequent constraints on a mother's ability to adjust litter sex ratios towards males. Maternal peri-conceptional testosterone, glucose, and glucocorticoids are the main hypothesised physiological mechanisms of maternal sex allocation (Figure 7.1). There is evidence to suggest that female testosterone, glucose, and glucocorticoids in adulthood are influenced by

prenatal testosterone and oestradiol exposure (Figure 7.1) (Eisner *et al.*, 2000; Padmanabhan *et al.*, 2010; Roland *et al.*, 2010; Ryan & Vandenbergh, 2002). As there is natural variation in the degree of feminisation in litter-bearing species, among a group of females, the physiological responses to sex allocation cues could then vary between females. Subsequently, litter sex ratio skews in response to sex allocation cues could be greater than or less than predicted by theory (see Figure 7.1). If, for example, feminised females' glucose regulation differs from more masculinised females (Cederroth & Nef, 2009; Roland *et al.*, 2010), then feminised and masculinised females could differ in the extent to which they adjust sex ratios according to factors that affect circulating glucose (e.g. resource availability, body condition; see Figure 7.1). Future work should test for such potential physiological constraints. If such physiological constraints on maternal sex allocation are widespread, mechanistic hypotheses of sex ratio adjustment would likely need to be adjusted to account for the physiological constraints prenatal testosterone and oestradiol exposure imposes on sex allocation for some mothers (Figure 7.1).

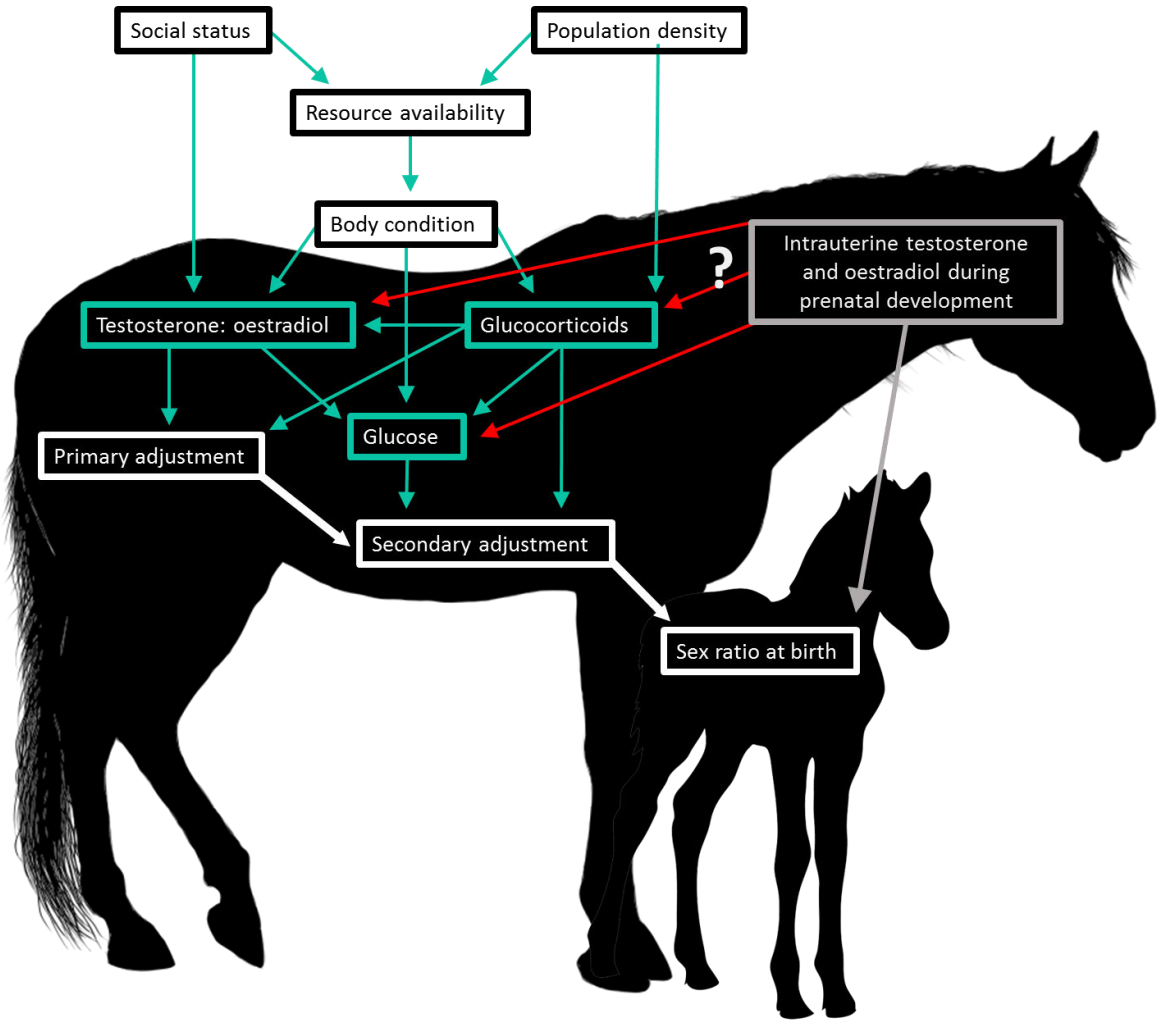

Figure 7.1. I showed that exposure to intrauterine testosterone and oestradiol during prenatal development (grey) influenced sex ratios at birth. However, the causal explanations (red) remain to be determined. If prenatal testosterone and oestradiol exposure affects sex ratios by influencing the physiological mechanisms (green) that are hypothesised to link cues of maternal sex allocation (black boxes) to sex ratio adjustment (white boxes), then not all females may be able to respond to cues of sex allocation to the extent theory predicts. Thus, there may be physiological constraints that mechanistic (green boxes) and theoretical (black boxes) hypotheses of sex allocation have not fully accounted for.

Here I identified the potential importance of prenatal feminisation on sex allocation for litter-bearing (polytocous) species (Chapter 2). I studied this relationship in a litter-bearing eutherian because of the added influence of testosterone and oestradiol from neighbouring siblings *in utero* contributing to inter-female variation in the degree of prenatal masculinisation and feminisation (Dušek, 2011; Ryan *et al*., 2002). However, other factors

also influence foetal exposure to testosterone and oestradiol. For example, genetic differences in endogenous testosterone and oestradiol production during prenatal development influence the degree to which an individual is masculinised or feminised (Warrington *et al.*, 2018). Furthermore, maternal conditions during gestation, such as social status, circulating glucocorticoids, exposure to endocrine-disrupting chemicals, and age also influence the degree to which a developing foetus is masculinised or feminised (Auger *et al.*, 2013; Barrett *et al.*, 2013; Baxter *et al.*, 2018; Kallak *et al.*, 2017; Lilley *et al.*, 2010; McLachlan *et al.*, 2006; Sachser & Kaiser, 1996). It is therefore likely that masculinising and feminising effects on sex ratios also occur in non-litter bearing (monotocous) eutherian mammals. Indeed, digit ratio has been shown to predict the sex of offspring in humans (Kim *et al.*, 2015; Manning *et al.*, 2002; Ventura *et al.*, 2013; but see Helle & Lilley, 2008), but has rarely been tested in other monotocous species, despite the potentially important implications for sex allocation. Since the selective advantages of maternal sex allocation are predicted to be greater for mothers that produce a single offspring at a time (Dušek, 2011; Myers, 1978), the consequences of the potential physiological constraints on maternal sex allocation I have suggested could be greater in monotocous species. Further work is required to clarify these potential physiological constraints in polytocous species, but should also include monotocous species to test whether the consequences of physiological constraints on sex allocation differ between these groups of mammals.

Current theoretical and mechanistic hypotheses of maternal sex allocation do not usually account for the influence of prenatal factors on maternal sex allocation and could therefore be missing an important explanatory variable in offspring sex ratios (Figure 7.1). Moreover, prenatal effects may further impose physiological constraints on maternal sex allocation. Sex

allocation hypotheses implicitly assume all females are equally capable of proximately modifying their offspring sex ratios (see West, 2009), which my work suggests is oversimplified. Thus, current sex allocation hypotheses would need to be reconsidered to account for the potential that not all females are able to adjust sex ratios in line with theoretical predictions.

Paternal influences on sex ratios

In the remainder of my thesis, I explored the potential influence of mammalian fathers on sex allocation (Chapters 3 to 6). Investigations into paternal influences on mammalian sex ratios to date have been limited including because sperm sex ratios have seldom been studied in more than one ejaculate from the same male (Chandler *et al.*, 2002; Chaudhary *et al.*, 2014; DeYoung *et al.*, 2004; Johannisson *et al.*, 2001). Thus, I identified the need to test the extent to which ejaculated sperm sex ratios vary within an individual male, particularly given the high inter-ejaculate variation in ejaculate composition (Perry *et al.*, 2013), as this would test the potential for paternal control over sperm sex ratios. In this thesis, I presented the first assessment of ejaculated sperm sex ratios and seminal plasma components with hypothesised sex-specific effects in multiple successive ejaculates of the same male in a mammalian species. Using horses (*Equus ferus caballus*) as a study species, I first showed that sperm sex ratios sometimes varied between successive ejaculates (Table 7.1; Chapter 3). I also found that seminal plasma zinc, an antioxidant, co-varied with sperm sex ratios in successive ejaculates (Table 7.1; Chapter 4). These results demonstrated that both sperm sex ratios and seminal fluid zinc vary between ejaculates from the same stallion, indicating proximal adjustment of sperm sex ratios and seminal fluid components with hypothesised

sex-specific effects do occur, thereby suggesting a potential role of fathers in influencing sex ratios. I then showed that the variation I observed in ejaculated sperm sex ratios in response to ejaculation frequency corresponded to variation in foal sex ratios in response to mating frequency in feral stallions (Chapter 5; Table 7.1), thereby linking proximal modification of sperm sex ratios to foal sex ratios. However, results were mixed (see Table 7.1 for mixed results and potential explanations), highlighting the complexity of proximal modifiers of sperm and offspring sex ratios and the need for further investigation clarifying when stallion mating frequency does and does not alter sperm and foal sex ratios (see Table 7.1).

Table 7.1. Summary of the results from Chapters 3 to 6 and plausible explanations for each result: a or b signify alternate hypotheses explaining the same result.

	Main findings	***Hypothesised explanations for results***
Chapter 3	*Population 1:* 1. More X-CBS in successive ejaculates as ejaculation frequency increased within three days *Population 2:* 2. X-CBS bias in ejaculates 3. No change in sperm sex ratio as ejaculation frequency increased within three days	*Population 1:* 1a) More rapid depletion Y-CBS stored in the epididymis when ejaculation frequency is increased to induce sexual exhaustion 1b) Greater allocation of X-CBS across multiple mating events with the same, sexually familiar, mares *Population 2:* 2. Lower seasonal ejaculation frequency or ejaculation occurring earlier and later in the season, both of which are linked to lower testosterone, contributed to consistent X-CBS bias 3. Shift in sperm sex ratio when ejaculation frequency is increased doesn't occur when seasonal mating frequency is low
Chapter 4	4. Increase in X-CBS corresponding to decrease in seminal magnesium 5. Proportion of Y-CBS positively correlated with seminal zinc	4. Seminal magnesium depleted by increased ejaculation frequency 5. Positive correlation between seminal zinc and Y-CBS: could represent a protective mechanism as Y-CBS are more susceptible to oxidative damage.
Chapter 5	6. Band stallions sired more daughters when there were more mares to mate during the week of conception	6a) More rapid depletion of Y-CBS stored in the epididymis when ejaculation frequency is increases 6b) Greater allocation of X-CBS across multiple mating events with same mares
Chapter 6	7. Daily, weekly, and seasonal mating frequency did not predict foal sex	7a) Extraordinarily high frequency provided little opportunity for sperm accumulation in epididymis to lead to differential depletion rates or other potential sex-specific effects during epididymal storage 7b) No variation in sperm sex ratios as each successive mating event was with different and sexually novel mares

My findings have important implications for mammalian sex allocation. Previous work has established that sperm sex ratios vary according to premating conditions, likely through physiological mechanisms occurring during sperm development and maturation (Figure 7.2) (e.g. Firman *et al*., 2020; Lavoie *et al.*, 2019; Tiido *et al.*, 2005; Van Hooft *et al.*, 2010). Here I showed that there are proximal modifiers of sperm sex ratios that likely influence offspring sex ratios. Moreover, I showed that zinc, a seminal plasma antioxidant, co-varied with sperm sex ratios (Chapter 4). Y-CBS are more vulnerable to oxidative damage (Oyeyipo *et al.*, 2017; You *et al.*, 2017) and therefore could be differentially influenced by variation in seminal antioxidant concentrations. If so, then variation in seminal plasma antioxidant composition could influence the sex of the resulting offspring. My results have demonstrated that there is inter-ejaculate variation in both sperm sex ratios and seminal fluid, and thus widen our understanding of paternal sex allocation to potentially include proximal modifiers of sperm sex ratios and seminal plasma composition (see Figure 7.2). These are exciting results that support further investigations. For example, does within-male variation in sperm sex ratios occur in a wide variety of species under a range of socio-sexual conditions or limited to certain circumstances in some species?

I suggest that male mating frequency likely influences sperm sex ratios in other mammalian species as well, particularly in highly polygynous species. In species where mating frequencies with multiple partners for both males and females are high, sperm competition and the risk sperm depletion for males is higher (Kvarnemo & Simmons, 2013; Møller & Birkhead, 1989; Preston *et al.*, 2001). Under such conditions, there may be high variability in sperm allocation and depletion patterns across multiple mating events (see Kvarnemo & Simmons, 2013; Parker & Pizzari, 2010; Parker & Ball, 2005; Preston *et al.*, 2001; Wedell *et al.*, 2002). I

suggested that the shift in sperm sex ratios recorded in Chapter 3 was linked to sperm depletion or allocation patterns across multiple mating events. It is therefore plausible that variation in sperm sex ratios relative to mating frequency is more likely to occur in species where both males and females mate at high frequencies with multiple partners. For example, if the observed shift in sperm sex ratios in Chapter 3 related to reduced sperm allocation to successive ejaculates with sexually familiar females (i.e. the Coolidge effect; Alvarez-Fernandez *et al.*, 2019; Dewsbury, 1981), this shift would likely not occur in monogamous species if males do not normally mate with multiple females (Dewsbury, 1981). Conversely, the variation in sperm sex ratios across multiple ejaculates might be greater in species like Soay sheep (*Ovis aries*), where both males and females mate with a high number of partners (Preston *et al.*, 2001), compared to stallions (Chapter 3) since horses exhibit female-defence polygyny, with the dominant band stallion monopolising mating with a limited number of mares (Ransom *et al.*, 2016). Comparing sperm sex ratio variation in, for example, the mostly monogamous prairie vole (*Microtus ochrogaster*; Young *et al.*, 2011) and the related but promiscuous meadow vole (*Microtus pennsylvanicus*; Boonstra *et al.*, 1993; McGuire *et al.*, 1992) would help to inform this hypothesis.

My results indicated that proximal modification of paternal sex ratios is plausible but further work is required to determine the extent to which fathers shift ejaculate composition to influence sex ratios and furthermore, whether shifts have adaptive implications. Males modify ejaculate composition, including sperm numbers and seminal plasma antioxidants, according to several aspects of the breeding environment including their mating role, the risk and intensity of sperm competition, and the number and quality of available females (Burger *et al.*, 2015b; Cornwallis and Birkhead, 2007; Cornwallis & O'Connor, 2009; Ferkin, 2004;

Jeannerat *et al.*, 2018; Joseph *et al.*, 2015; Kilgallon & Simmons, 2005; Lemaitre *et al.*, 2012; Mora *et al.*, 2017; Perry *et al*., 2013. Ramm *et al.*, 2015). There is, therefore, inter-ejaculate variation in seminal plasma composition as well as variation in sperm allocation per ejaculate that my results suggested might facilitate variation in ejaculate sperm sex ratios (Chapter 3 & 4). Manipulating socio-sexual factors known to produce adaptive responses in ejaculate composition could test the effect of proximal modifiers on paternal sex allocation (see Figure 7.2). Recently, perceived male mate competition during sexual development has been linked to skewed production of X- and Y-CBS, suggesting proximal mating cues to paternal sex allocation (Firman *et al.*, 2020; Lavoie *et al.*, 2019). However, male proximal modification of sperm sex ratios in response to mate competition remains to be tested. As stallions show variation in ejaculate composition (including seminal plasma composition) in response to the presence of rival stallions and the attractiveness of the mare (Burger *et al.*, 2015a; Burger *et al.*, 2015b; Jeannerat *et al.*, 2018), as well as variation in sperm sex ratios between ejaculates (Chapter 3 & 4), an informative next step would be to assess ejaculated sperm sex ratios and seminal plasma composition as exposure to mares and other stallions is manipulated.

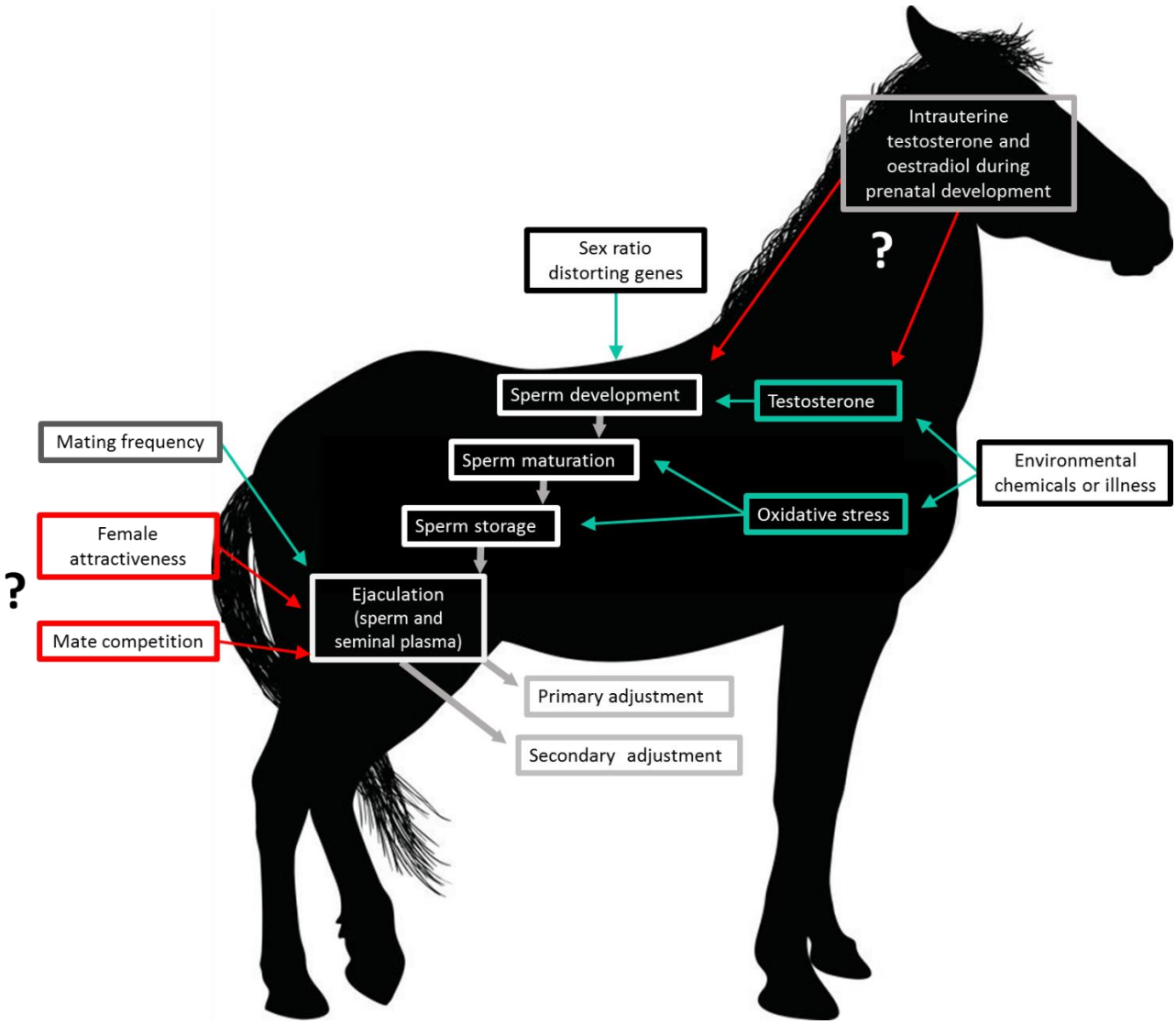

Figure 7.2. The results of my work showed that sperm sex ratios varied according to proximal breeding conditions, specifically mating frequency (dark grey). Thus, the sperm sex ratio in an ejaculate is likely the result of both premating factors (black boxes) that influence (green) sperm development and maturation (white boxes) and proximal modifiers of sperm sex ratios. Further investigation is required to determine whether there are other proximal modifiers of paternal sex ratios (red). Prenatal effects (grey) might also influence sperm sex ratios, potentially presenting physiological constraints on paternal sex allocation.

I previously discussed potential physiological constraints on maternal sex allocation relating to a female's prenatal testosterone and oestradiol exposure. Future work in paternal sex allocation should also consider the possibility of physiological constraints on paternal ability to proximately modify sperm sex ratios (Figure 7.2). Male mice from male-biased litters

produced more Y-CBS compared to males from female-biased litters (Lavoie *et al*., 2019), suggesting that there are prenatal masculinising and feminising effects on sperm sex ratios, as well as on maternal sex allocation (Chapter 2). Thus, it is plausible that masculinised males might show reduced shifts towards X-CBS when, for example, mating frequency is increased (Chapter 3) or male conspecific density is decreased (Lavoie *et al*., 2019). Exposing prenatally feminised males to conditions that predict higher Y-CBS and vice versa will help to assess this hypothesis. Digit ratio would also be a useful measure to account for potential prenatal effects in future paternal sex allocation for some species.

Some of my findings linked proximal modification of sperm sex ratios to foal sex ratio variation (Chapter 3, 4, & 5), suggesting that fathers not only adjust sperm sex ratios according to their current condition but also influence offspring sex ratios. Since mammalian mothers also influence sex ratios, paternal and maternal effects likely interact. Since male and female interests will not always align, these interactions could be complementary, antagonistic, or neutral (see Arnqvist & Rowe, 2005; Chapman, 2006; Edwards & Cameron, 2014; Foerster *et al.*, 2007; King, 2010; Lynch and Cronk, 2018). Therefore, differences in the direction of maternal and paternal effects could produce inconsistent results if only one parent is considered in a study. For example, opposing maternal and paternal sex ratio adjustment has recently been implicated in tammar wallabies (*Macropus eugenii;* Edwards *et al.*, 2019), suggesting that opposing parental effects could help explain why biases are sometimes not observed. Alternatively, if the direction of maternal and paternal sex allocation aligns, sex ratio skews might be enhanced (see Douhard, 2018).

In Chapter 5 and 6, the sperm sex ratio in each mating event was not known. I therefore could not determine if foal sex ratios in Chapter 5 were related to sperm sex ratio adjustment or rather a maternal effect. I also could not determine whether stallions in Chapter 6 showed no variation in sperm sex ratios or whether sperm sex ratio skews were masked by an antagonistic maternal effect. Such results highlight the importance of assessing both maternal and paternal influences on offspring sex ratios and their potential interactions. Measuring the sperm sex ratios and seminal plasma composition of ejaculates received by females for whom diet or hormones have been manipulated to influence sex ratios (e.g. Helle *et al.*, 2008; Rosenfeld & Roberts, 2004) would help to indicate how maternal and paternal sex allocation constrain or enhance each other. For example, does an X-CBS bias in the ejaculate reduce the likelihood of mothers with experimentally elevated glucose having sons? Understanding such interactions has significant implications for the current understanding of sex allocation. For example, if parents skew sex ratios in opposing directions, this could lead to constraints on the ability of some parents to adjust sex ratios to the extent predicted by theory.

Conclusions

Interpretations of sex allocation that incorporate both maternal and paternal factors, including the potential physiological constraints suggested by my work, will aid reconciliation of the sometimes inconsistent empirical results. While mammalian mothers clearly can and do adjust sex ratios according to sex allocation cues, some mothers may be constrained in their ability to do so. Moreover, my results broaden our understanding of paternal sex allocation. I provided evidence that sperm sex ratios and seminal plasma components with

hypothesised sex-specific effects vary between ejaculates from the same male according to the proximal breeding environment, thereby indicating the potential for paternal influence over sex ratios. I also found evidence linking proximal modification of sperm sex ratios to variation in offspring sex ratios, further highlighting this potential. Further work is required to clarify potential proximal cues of paternal sex allocation and determine how widespread this variation is. However, a greater consideration for fathers is essential in future mammalian sex allocation research. Explanations of sex ratios that include the interests of and constraints on both mothers and fathers will provide a more complete picture of mammalian sex allocation.

References

Aloise King, E. D., Banks, P. B., & Brooks, R. C. (2013). Sexual conflict in mammals: consequences for mating systems and life history. *Mammal Review*, *43*, 47-58.

Alvarez-Fernandez, A., Borziak, K., McDonald, G. C., Dorus, S., & Pizzari, T. (2019). Female novelty and male status dynamically modulate ejaculate expenditure and seminal plasma proteome over successive matings in red junglefowl. *Scientific Reports, 9*, 5852.

Arnqvist, G., & Rowe, L. (2005). *Sexual Conflict*. Princeton, USA: Princeton University Press.

Auger, J., Le Denmat, D., Berges, R., Doridot, L., Salmon, B., Canivenc-Lavier, M. C., & Eustache, F. (2013). Environmental levels of oestrogenic and antiandrogenic compounds feminize digit ratios in male rats and their unexposed male progeny. *Proceedings of the Royal Society B: Biological Sciences, 280*, 20131532.

Bánszegi, O., Szenczi, P., Dombay, K., Bilkó, Á., & Altbäcker, V. (2012). Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. *Physiology & Behavior, 105*, 1226-1230.

Barrett, E. S., Parlett, L. E., Sathyanarayana, S., Liu, F., Redmon, J. B., Wang, C., & Swan, S. H. (2013). Prenatal exposure to stressful life events is associated with masculinized anogenital distance (AGD) in female infants. *Physiology & Behavior, 114*, 14-20.

Bartlett, M. J., Steeves, T. E., Gemmell, N. J., & Rosengrave, P. C. (2017). Sperm competition risk drives rapid ejaculate adjustments mediated by seminal plasma. *Elife, 6*, e28811.

Baxter, A., Wood, E. K., Jarman, P., Cameron, A. N., Capitanio, J. P., & Higley, J. D. (2018). Sex differences in rhesus monkeys' digit ratio (2D: 4D ratio) and its association with maternal social dominance rank. *Frontiers in Behavioral Neuroscience, 12*, 1-12.

Boonstra, R., Xia, X., & Pavone, L. (1993). Mating system of the meadow vole, *Microtus pennsylvanicus*. *Behavioral Ecology, 4*, 83-89.

Brown, W. M., Finn, C. J., & Breedlove, S. M. (2002). Sexual dimorphism in digit-length ratios of laboratory mice. *The Anatomical Record, 267*, 231-234.

Burger, D., Dolivo, G., Marti, E., Sieme, H., & Wedekind, C. (2015a). Female major histocompatibility complex type affects male testosterone levels and sperm number in the horse (*Equus caballus*). *Proceedings of the Royal Society B: Biological Sciences, 282*, 20150407.

Burger, D., Dolivo, G., & Wedekind, C. (2015b). Ejaculate characteristics depend on social environment in the horse (*Equus caballus*). *PLoS One, 10*, e0143185.

Cameron, E., Day, T., & Rowe, L. (2007). Sperm competition and the evolution of ejaculate composition. *The American Naturalist, 169*, 158-172.

Cameron, E. Z., Lemons, P. R., Bateman, P. W., & Bennett, N. C. (2008). Experimental alteration of litter sex ratios in a mammal. *Proceedings of the Royal Society B: Biological Sciences, 275*, 323-327.

Chandler, J. E., Canal, A. M., Paul, J. B., & Moser, E. B. (2002). Collection frequency affects percent Y-chromosome-bearing sperm, sperm head area and quality of bovine ejaculates. *Theriogenology, 57*, 1327-1346.

Chapman, T. (2006). Evolutionary conflicts of interest between males and females. *Current Biology, 16*, 744-754.

Chaudhary, I., Jain, M., & Halder, A. (2014). Sperm sex ratio (X: Y Ratio) and its variations. *Austin Journal of Reproductive Medicine & Infertility, 1*, 1-7.

Cederroth, C. R., & Nef, S. (2009). Fetal programming of adult glucose homeostasis in mice. *PLoS One*, *4*, e7281.

Cohas, A., & Allainé, D. (2009). Social structure influences extra-pair paternity in socially monogamous mammals. *Biology Letters, 5*, 313-316.

Cornwallis, C. K., & Birkhead, T. R. (2007). Changes in sperm quality and numbers in response to experimental manipulation of male social status and female attractiveness. *The American Naturalist*, *170*, 758-770.

Cornwallis, C. K., & O'Connor, E. A. (2009). Sperm: seminal plasma interactions and the adjustment of sperm quality in relation to female attractiveness. *Proceedings of the Royal Society B: Biological Sciences, 276*, 3467-3475.

Correa, L. A., León, C., Ramírez-Estrada, J., Soto-Gamboa, M., Sepulveda, R. D., & Ebensperger, L. A. (2016). Masculinized females produce heavier offspring in a group living rodent. *Journal of Animal Ecology, 85*, 1552-1562.

Dewsbury, D. A. (1981). Effects of novelty of copulatory behavior: the Coolidge effect and related phenomena. *Psychological Bulletin, 89*, 464-482.

DeYoung, R. W., Muller, L. I., Demarais, S., Guthrie, H. D., Welch, G. R., Engelken, T. J., & Gonzales, R. A. (2004). Do *Odocoileus virginianus* males produce Y-chromosome-biased ejaculates? Implications for adaptive sex ratio theories. *Journal of Mammalogy, 85*, 768-773.

Douhard, M. (2018). The role of fathers in mammalian sex allocation. *Mammal Review, 48*, 67-74.

Dušek, A. (2011). *Maternal Investment Strategy in Model Monotocous and Polytocous Mammals: A Life-History Perspective.* (Doctoral thesis). Univerzita Karlova, Prague.

Dušek, A., Bartoš, L., & Sedláček, F. (2010). Developmental instability of ano-genital distance index: implications for assessment of prenatal masculinization. *Developmental psychobiology*, *52*, 568-573.

Edwards, A. M., & Cameron, E. Z. (2014). Forgotten fathers: paternal influences on mammalian sex allocation. *Trends in Ecology & Evolution, 29*, 158-164.

Edwards, A. M., Cameron, E. Z., Deakin, J. E., Ezaz, T., Pereira, J. C., Ferguson-Smith, M. A., & Robert, K. A. (2019). Sexual conflict in action: an antagonistic relationship between maternal and paternal sex allocation in the tammar wallaby, *Notamacropus eugenii*. *Ecology and Evolution, 9*, 4340-4348.

Eisner, J. R., Dumesic, D. A., Kemnitz, J. W., & Abbott, D. H. (2000). Timing of prenatal androgen excess determines differential impairment in insulin secretion and action in adult female rhesus monkeys. *The Journal of Clinical Endocrinology & Metabolism, 85*, 1206-1210.

Ferkin, M. H. (2004). Male mammals respond to a risk of sperm competition conveyed by odours of conspecific males. *Nature, 431*, 446-449.

Firman, R. C., Klemme, I., & Simmons, L. W. (2013). Strategic adjustments in sperm production within and between two island populations of house mice. *Evolution, 67*, 3061-3070.

Firman, R. C., Tedeschi, J. N., & Garcia-Gonzalez, F. (2020). Sperm sex ratio adjustment in a mammal: perceived male competition leads to elevated proportions of female-producing sperm. *Biology Letters, 16*, 20190929.

Foerster, K., Coulson, T., Sheldon, B. C., Pemberton, J. M., Clutton-Brock, T. H., & Kruuk, L. E. (2007). Sexually antagonistic genetic variation for fitness in red deer. *Nature, 447*, 1107-1110.

Gomendio, M., Malo, A. F., Soler, A. J., Fernández-Santos, M. R., Esteso, M. C., García, A. J., Roldan, E. R., & Garde, J. (2006). Male fertility and sex ratio at birth in red deer. *Science, 314*, 1445-1447.

Grant, V. J. (2007). Could maternal testosterone levels govern mammalian sex ratio deviations? *Journal of Theoretical Biology, 246*, 708-719.

Helle, S., Laaksonen, T., Adamsson, A., Paranko, J., & Huitu, O. (2008). Female field voles with high testosterone and glucose levels produce male-biased litters. *Animal Behaviour, 75*, 1031-1039.

Helle, S., & Lilley, T. (2008). Maternal 2nd to 4th digit ratio does not predict lifetime offspring sex ratio at birth. *American Journal of Human Biology, 20*, 700-703.

Hickey, M., Doherty, D., Hart, R., Norman, R., Mattes, E., Atkinson, H., & Sloboda, D. (2010). Maternal and umbilical cord androgen concentrations do not predict digit ratio (2D: 4D) in girls: a prospective cohort study. *Psychoneuroendocrinology, 35*, 1235-1244.

Hollier, L. P., Keelan, J. A., Jamnadass, E. S., Maybery, M. T., Hickey, M., & Whitehouse, A. J. (2015). Adult digit ratio (2D: 4D) is not related to umbilical cord androgen or estrogen concentrations, their ratios or net bioactivity. *Early Human Development, 91*, 111-117.

Hu, M., Richard, J. E., Maliqueo, M., Kokosar, M., Fornes, R., Benrick, A., Jansson, T., Ohlsson, C., Wu, X., & Skibicka, K. P. (2015). Maternal testosterone exposure increases anxiety-like behavior and impacts the limbic system in the offspring. *Proceedings of the National Academy of Sciences, 112*, 14348-14353.

Hurd, P. L., Bailey, A. A., Gongal, P. A., Yan, R. H., Greer, J. J., & Pagliardini, S. (2008). Intrauterine position effects on anogenital distance and digit ratio in male and female mice. *Archives of Sexual Behavior, 37*, 9-18.

Jeannerat, E., Janett, F., Sieme, H., Wedekind, C., & Burger, D. (2017). Quality of seminal plasmas varies with type of stimulus at ejaculation. *Scientific Reports, 7*, 44339.

Jeannerat, E., Marti, E., Berney, C., Janett, F., Bollwein, H., Sieme, H., Burger, D., & Wedekind, C. (2018). Stallion semen quality depends on major histocompatibility complex matching to teaser mare. *Molecular Ecology, 27*, 1025-1035.

Johannisson, R., Leuschner, E., Hüppe, M., Hinrichs, F., Al-Hasani, S., Diedrich, K., Schwinger, E., & Mennicke, K. (2001). Increased frequency of X-bearing sperm in males from an infertility clinic: analysis by two-color fluorescence *in situ* hybridization. *Cytogenetic and Genome Research, 98*, 240-244.

Johnson, L., Varner, D. D., Roberts, M. E., Smith, T. L., Keillor, G. E., & Scrutchfield, W. L. (2000). Efficiency of spermatogenesis: a comparative approach. *Animal Reproduction Science*, *60*, 471-480.

Joseph, P. N., Sharma, R. K., Agarwal, A., & Sirot, L. K. (2015). Men ejaculate larger volumes of semen, more motile sperm, and more quickly when exposed to images of novel women. *Evolutionary Psychological Science, 1*, 195-200.

Kallak, T. K., Hellgren, C., Skalkidou, A., Sandelin-Francke, L., Ubhayasekhera, K., Bergquist, J., Axelsson, O., Comasco, E., Campbell, R. E., & Poromaa, I. S. (2017). Maternal and female fetal testosterone levels are associated with maternal age and gestational weight gain. *European Journal of Endocrinology, 177*, 379-388.

Kvarnemo, C., & Simmons, L. W. (2013). Polyandry as a mediator of sexual selection before and after mating. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*, 20120042.

Kilgallon, S. J., & Simmons, L. W. (2005). Image content influences men's semen quality. *Biology Letters, 1*, 253-255.

Kim, T. B., Oh, J. K., Kim, K. T., Yoon, S. J., & Kim, S. W. (2015). Does the mother or father determine the offspring sex ratio? Investigating the relationship between maternal digit ratio and offspring sex ratio. *PLoS One, 10*, e0143054.

Kita, D. H., Meyer, K. B., Venturelli, A. C., Adams, R., Machado, D. L., Morais, R. N., ... & Martino-Andrade, A. J. (2016). Manipulation of pre and postnatal androgen environments and anogenital distance in rats. *Toxicology*, *368*, 152-161.

Lavoie, M. D., Tedeschi, J. N., Garcia-Gonzalez, F., & Firman, R. C. (2019). Exposure to male-dominated environments during development influences sperm sex ratios at sexual maturity. *Evolution Letters, 3*, 392-402.

Lemaitre, J. F., Ramm, S. A., Hurst, J. L., & Stockley, P. (2012). Sperm competition roles and ejaculate investment in a promiscuous mammal. *Journal of Evolutionary Biology, 25*, 1216-1225.

Lilley, T., Laaksonen, T., Huitu, O., & Helle, S. (2010). Maternal corticosterone but not testosterone level is associated with the ratio of second-to-fourth digit length (2D: 4D) in field vole offspring (*Microtus agrestis*). *Physiology & Behavior, 99*, 433-437.

Lutchmaya, S., Baron-Cohen, S., Raggatt, P., Knickmeyer, R., & Manning, J. T. (2004). 2nd to 4th digit ratios, fetal testosterone and estradiol. *Early Human Development, 77*, 23-28.

Lynch, R., Wasielewski, H., & Cronk, L. (2018). Sexual conflict and the Trivers-Willard hypothesis: females prefer daughters and males prefer sons. *Scientific Reports*, *8*, 1-13.

Malo, A. F., Martinez-Pastor, F., Garcia-Gonzalez, F., Garde, J., Ballou, J. D., & Lacy, R. C. (2017). A father effect explains sex-ratio bias. *Proceedings of the Royal Society B: Biological Sciences, 284*, 20171159.

Manning, J. T. (2011). Resolving the role of prenatal sex steroids in the development of digit ratio. *Proceedings of the National Academy of Sciences, 108*, 16143-16144.

Manning, J. T., Martin, S., Trivers, R. L., & Soler, M. (2002). 2nd to 4th digit ratio and offspring sex ratio. *Journal of Theoretical Biology, 217*, 93-95.

McGuire, B., Drewsen Russell, K., Mahoney, T., & Novak, M. (1992). The effects of mate removal on pregnancy success in prairie voles (*Microtus ochrogaster*) and meadow voles (*Microtus pennsylvanicus*). *Biology of Reproduction, 47*, 37-42.

McLachlan, J. A., Simpson, E., & Martin, M. (2006). Endocrine disrupters and female reproductive health. *Best Practice & Research Clinical Endocrinology & Metabolism, 20*, 63-75.

Mitchell, R. T., Mungall, W., McKinnell, C., Sharpe, R. M., Cruickshanks, L., Milne, L., & Smith, L. B. (2015). Anogenital distance plasticity in adulthood: implications for its use as a biomarker of fetal androgen action. *Endocrinology*, *156*, 24-31.

Møller, A., & Birkhead, T. (1989). Copulation behaviour in mammals: evidence that sperm competition is widespread. *Biological Journal of the Linnean Society, 38*, 119-131.

Monclús, R., & Blumstein, D. T. (2012). Litter sex composition affects life-history traits in yellow-bellied marmots. *Journal of Animal Ecology, 81*, 80-86.

Moore, A. M., Prescott, M., & Campbell, R. E. (2013). Estradiol negative and positive feedback in a prenatal androgen-induced mouse model of polycystic ovarian syndrome. *Endocrinology, 154*, 796-806.

Mora, A. R., Meniri, M., Gning, O., Glauser, G., Vallat, A., & Helfenstein, F. (2017). Antioxidant allocation modulates sperm quality across changing social environments. *PLoS One, 12*, e0176385.

Myers, J. H. (1978). Sex ratio adjustment under food stress: maximization of quality or numbers of offspring? *The American Naturalist, 112*, 381-388.

Nelson, E., Hoffman, C. L., Gerald, M. S., & Shultz, S. (2010). Digit ratio (2D: 4D) and dominance rank in female rhesus macaques (*Macaca mulatta*). *Behavioral Ecology and Sociobiology, 64*, 1001-1009.

Oyeyipo, I. P., van der Linde, M., & du Plessis, S. S. (2017). Environmental exposure of sperm sex-chromosomes: a gender selection technique. *Toxicological Research, 33*, 315-323.

Padmanabhan, V., Veiga-Lopez, A., Abbott, D., Recabarren, S., & Herkimer, C. (2010). Developmental programming: impact of prenatal testosterone excess and postnatal weight gain on insulin sensitivity index and transfer of traits to offspring of overweight females. *Endocrinology, 151*, 595-605.

Parker, G. A., & Ball, M. A. (2005). Sperm competition, mating rate and the evolution of testis and ejaculate sizes: a population model. *Biology Letters, 1*, 235-238.

Parker, G. A., & Pizzari, T. (2010). Sperm competition and ejaculate economics. *Biological Reviews, 85*, 897-934.

Perret, M. (2019). Litter sex composition affects first reproduction in female grey mouse lemurs (*Microcebus murinus*). *Physiology & Behavior, 208*, 112575.

Perry, J. C., Sirot, L., & Wigby, S. (2013). The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution, 28*, 414-422.

Preston, B. T., Stevenson, I. R., Pemberton, J. M., & Wilson, K. (2001). Dominant rams lose out by sperm depletion. *Nature, 409*, 681-682.

Ramm, S. A., Edward, D. A., Claydon, A. J., Hammond, D. E., Brownridge, P., Hurst, J. L., Beynon, R. J., & Stockley, P. (2015). Sperm competition risk drives plasticity in seminal plasma composition. *BMC Biology, 13*, 1-18.

Ransom, J. I., & Kaczensky, P. (Eds.). (2016). *Wild Equids: Ecology, Management, and Conservation*. Baltimore, USA: Johns Hopkins University Press.

Roland, A. V., Nunemaker, C. S., Keller, S. R., & Moenter, S. M. (2010). Prenatal androgen exposure programs metabolic dysfunction in female mice. *Journal of Endocrinology, 207*, 213-223.

Rosenfeld, C. S., & Roberts, R. M. (2004). Maternal diet and other factors affecting offspring sex ratio: a review. *Biology of Reproduction, 71*, 1063-1070.

Ryan, B. C., & Vandenbergh, J. G. (2002). Intrauterine position effects. *Neuroscience & Biobehavioral Reviews, 26*, 665-678.

Ryan, C. P., Anderson, W. G., Gardiner, L. E., & Hare, J. F. (2012). Stress-induced sex ratios in ground squirrels: support for a mechanistic hypothesis. *Behavioral Ecology, 23*, 160-167.

Sachser, N., & Kaiser, S. (1996). Prenatal social stress masculinizes the females' behaviour in guinea pigs. *Physiology & Behavior, 60*, 589-594.

Schwartz, C. L., Christiansen, S., Vinggaard, A. M., Axelstad, M., Hass, U., & Svingen, T. (2019). Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Archives of Toxicology*, *93*, 253-272.

Sullivan, S. D., & Moenter, S. M. (2004). Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. *Proceedings of the National Academy of Sciences, 101*, 7129-7134.

Szenczi, P., Banszegi, O., Groo, Z., & Altbäcker, V. (2013). Anogenital distance and condition as predictors of litter sex ratio in two mouse species: a study of the house mouse (*Mus musculus*) and mound-building mouse (*Mus spicilegus*). *PLoS One, 8*, e74066.

Tiido, T., Rignell-Hydbom, A., Jönsson, B., Giwercman, Y. L., Rylander, L., Hagmar, L., & Giwercman, A. (2005). Exposure to persistent organochlorine pollutants associates with human sperm Y: X chromosome ratio. *Human Reproduction, 20*, 1903-1909.

Van Hooft, P., Prins, H. H., Getz, W. M., Jolles, A. E., van Wieren, S. E., Greyling, B. J., van Helden, P. D., & Bastos, A. D. (2010). Rainfall-driven sex-ratio genes in African buffalo suggested by correlations between Y-chromosomal haplotype frequencies and foetal sex ratio. *BMC Evolutionary Biology, 10*, 1-11.

Ventura, T., Gomes, M., Pita, A., Neto, M., & Taylor, A. (2013). Digit ratio (2D: 4D) in newborns: influences of prenatal testosterone and maternal environment. *Early Human Development, 89*, 107-112.

Vreeburg, J., Groeneveld, J., Post, P., & Ooms, M. (1983). Concentrations of testosterone and androsterone in peripheral and umbilical venous plasma of fetal rats. *Journal of Reproduction and Fertility, 68*, 171-175.

Warrington, N. M., Shevroja, E., Hemani, G., Hysi, P. G., Jiang, Y., Auton, A., Boer, C. G., Mangino, M., Wang, C. A., & Kemp, J. P. (2018). Genome-wide association study identifies nine novel loci for 2D: 4D finger ratio, a putative retrospective biomarker of testosterone exposure *in utero*. *Human Molecular Genetics, 27*, 2025-2038.

Wedell, N., Gage, M. J., & Parker, G. A. (2002). Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution, 17*, 313-320.

West, S. (2009). *Sex Allocation*. Princeton, USA: Princeton University Press.

Witham, E. A., Meadows, J. D., Shojaei, S., Kauffman, A. S., & Mellon, P. L. (2012). Prenatal exposure to low levels of androgen accelerates female puberty onset and reproductive senescence in mice. *Endocrinology, 153*, 4522-4532.

You, Y. A., Kwon, W. S., Saidur Rahman, M., Park, Y. J., Kim, Y. J., & Pang, M. G. (2017). Sex chromosome-dependent differential viability of human spermatozoa during prolonged incubation. *Human Reproduction, 32*, 1183-1191.

Young, K. A., Gobrogge, K. L., Liu, Y., & Wang, Z. (2011). The neurobiology of pair bonding: insights from a socially monogamous rodent. *Frontiers in Neuroendocrinology, 32*, 53-69.

Zheng, Z., & Cohn, M. J. (2011). Developmental basis of sexually dimorphic digit ratios. *Proceedings of the National Academy of Sciences, 108*, 16289-16294.