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It’s your hormones, deer.

Individual variation in hormone levels within a wild population of red deer: causes and consequences.

Alyson Pavitt

Submitted for the degree of Doctor of Philosophy

The University of Edinburgh

2015

Abstract

Whilst individual differences in circulating hormone levels can influence life history traits throughout an animal's lifetime, this remains a poorly understood area of research, particularly for wild systems where sufficient sets of individual-based data are rare. This thesis aimed to address this dearth of information by identifying key drivers of hormone variation, as well as exploring potential fitness consequences within a single system of wild red deer (*Cervus elaphus*) on the Isle of Rum National Nature Reserve in Scotland. It focussed on both androgen (e.g. testosterone) and glucocorticoid (e.g. cortisol) levels, and examined among-individual variation in these two hormone groups from samples collected using both traditional (blood: chapters 3 & 4) and non-invasive (faecal: chapters 5 & 6) methods.

Results showed both intrinsic and extrinsic factors to influence an individual's hormone levels. In general, current or recent environment explained the greatest variation, with both hormone groups exhibiting strong temporal trends at multiple scales. Concentrations changed substantially across an individual's lifetime as they aged (chapters 5 & 6), and calves born in different years differed in their neonatal testosterone levels (chapter 3). Hormone levels also varied across the year, showing clear seasonal cycles which peaked during key reproductive events: the calving season in females (chapter 6) and the rut in males (chapter 5). An individual's current life history state was also important, particularly a female's reproductive state (chapter 6). Whilst there was some evidence of maternal effects on neonatal hormone levels (chapter 3) these were not extensive, and maternal hormone concentrations did not appear to influence those in their new-born calves (chapter 6). There was, however, evidence of neonatal circulating testosterone levels being heritable, and despite overall differences between the sexes the underlying genetic architecture of this trait did not differ between male and female calves (chapter 4).

Associations were also found between an individual's hormone levels and their fitness, although these consequences were only apparent in short-term fitness measures or proxies such as reproductive behaviour (e.g. male reproductive effort

in chapter 5). Effects were also not ubiquitous within the population. Whilst a calf's circulating testosterone levels indicated their probability of surviving their first year of life, these effects were only apparent in firstborn males, a group which is particularly vulnerable to mortality (chapter 3). In general, this thesis suggests that the fitness consequences identified by broad-scale hormone manipulation studies can still be found when looking at subtle individual-level differences. The limited evidence of persistent hormone phenotypes (indicated by the lack of among-individual variance for most measures, chapter 5 & 6) does, however, emphasise the importance of repeatedly sampling individuals before drawing extensive conclusions about fitness consequences.

Lay summary

Hormones are chemical molecules produced in the body that play an integral role in many biological processes. Despite knowing that hormone levels can vary substantially between individual animals at any stage of life, the causes and consequences of this variation remain poorly understood in the wild. In this thesis I look at both androgens (e.g. testosterone) and stress hormones (e.g. cortisol) within a population of wild red deer (*Cervus elaphus*), and ask (a) what causes levels of these hormones to differ within and between individuals, and (b) whether this could impact on their fitness.

In this thesis, I show the importance of understanding how and why individuals differ in their hormone concentrations, and the necessity of being able to account for specific causes of that variation when exploring fitness-linked consequences. An individual's hormone levels at a given point in time are due to the combined effect of their environment and their intrinsic state. In general, current or recent environment explained the greatest variation, with both androgen and cortisol exhibiting strong temporal trends at multiple scales. Early life testosterone levels varied between calves born in different years, whilst adult androgen and cortisol levels exhibited clear seasonal cycles, peaking during key reproductive events: the spring calving season in females and the autumn rut in males. Age also had a strong effect on an individual's measurable hormone levels: testosterone levels declined rapidly within the first day of life, cortisol levels increased with age, and adult androgen levels peaked in middle-aged stags. An individual's current life history state also appeared to be important, particularly whether females were pregnant or lactating. Whilst there was some evidence of maternal effects on calf hormone levels, these were minimal, and a mother's hormone concentrations did not appear to influence those in their new-born calves.

There were also associations between an individual's hormone levels and components of fitness, although these were only apparent amongst short-term fitness measures or proxies such as reproductive behaviour. Effects were not,

however, ubiquitous within the population, and the limited evidence of repeatable hormone concentrations within an individual deer emphasises the importance of repeatedly sampling individuals. Whilst a calf's testosterone levels indicated their probability of surviving their first year of life, these effects were only apparent in firstborn males, a group which is particularly vulnerable to mortality. This suggests that the fitness consequences identified by broad-scale hormone manipulation studies can still be found when looking at individual-level differences, though effects may be subtle and not ubiquitous. The analyses presented in this thesis ultimately show that hormone levels can vary hugely between individuals, and far from just being error around a population mean, this variation is incredibly valuable in better understanding a wild population's ecology.

Declaration

The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The long-term data, and many of the biological samples, used in this thesis were collected by others as part of the long-running Rum red deer project. I did, however, contribute to sample collection and carried out the laboratory analyses necessary to obtain hormone measures from all the samples used (with the exception of the validation assays presented in chapter 2.5 which were run in collaboration with Prof. E. Moestl's lab at the University of Veterinary Medicine, Vienna). Throughout my PhD, all three supervisors (Prof. L. Kruuk, Dr. C. Walling, and Prof. J. Pemberton) contributed to discussions on biological interpretation, and provided analytical advice. Ultimately, however, I wrote the thesis and carried out all the analyses presented in the following chapters (and in the publications listed below).

Chapters 3-6 all form the basis of papers either in publication (chapters 3 & 4), in press (chapter 5) or in review (chapter 6) at the time of thesis submission (see publication list below). Feedback from supervisors, and from other authors on specific papers/manuscripts have been incorporated throughout the text: Prof. A. McNeilly (Chapter 3) and Prof. E. Moestl (Chapter 5).

Manuscript/ publications

Pavitt, A., C. Walling, J. Pemberton, and L. Kruuk. 2014a. Causes and consequences of variation in early life testosterone in a wild population of red deer. Functional Ecology **28**:1224-1234.

Pavitt, A., C. Walling, J. Pemberton, and L. Kruuk. 2014b. Heritability and cross-sex genetic correlations of early-life circulating testosterone levels in a wild mammal. Biology Letters **10**.

Pavitt, A., C. Walling, E. Mostl, J. Pemberton, and L. Kruuk. In press. Cortisol but not testosterone is repeatable and varies with reproductive effort in wild red deer stags. General and Comparative Endocrinology.
Pavitt, A., C. Walling, J. Pemberton, and L. Kruuk. In review. Testosterone and cortisol concentrations vary with reproductive status in wild female red deer. Ecology and Evolution.

3/7/15

Acknowledgements

I owe a huge debt of thanks to my three supervisors, Loeske Kruuk, Josephine Pemberton, and Craig Walling for their tireless help, guidance, support and cheerleading. Thank you, Loeske, for your continued energy and commitment despite the distance, and Josephine and Craig for adopting me and my day-to-day upkeep. I am hugely grateful to you all for giving me the freedom to grow and experiment and find my own way through this PhD (and then for being there when I go off course).

None of this would have been possible without the dedication and knowledge of a huge range of staff and volunteers (both past and present) who have worked as part of the Rum red deer project. I particularly want to thank Ali Morris and Martyn Baker for making me feel so welcome on my visits to Rum, and for their tireless poo-collecting endeavours over the years. I also want to thank the rum red deer group (and affiliates) for useful discussions and comments. Finally, I'm immensely grateful to Forbes Howie (Centre for Reproductive Health, University of Edinburgh) and Erich Moestl (University of Veterinary Medicine, Vienna) for all their practical advice, help and enthusiasm in my endocrine pursuits.

Doing a PhD is not a solitary endeavour, and I would definitely have struggled were it not for my peers and friends in Edinburgh. A huge big sparkly thank you for making my time here so much fun, for eating my cake, for plying me with (lots of) tea, for being supportive and patient and for picking me up when I do something silly. You are all awesome! Rebecca, who has moved through the PhD ranks with me (we did it!); all my officemates and friends: Becky, Maarit, Kara, Mizuho, Elisa, and particularly Christine who has put up with so very much (and always has the perfect postcard on hand to make my day); and Caroline, who has been a great source of distraction, particularly in these last weeks. Thank you so much for babysitting me. Thank you to my long- (and short-) suffering flatmates Tom, Jess, and Georgina for providing fun distractions, debates, and philosophical musings to counter the rantings and the cake. To my family for your tireless support,

encouragement and hugs, and for humouring me when I explode science at you. And finally to Aaron who has been with me every step of the way. Thank you for the late night calls, the scientific debates, the baby animal pictures, and your endless, endless patience. This would have been so much harder without you all.

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III | Abbreviations

ACTH: Adrenocorticotropic hormone

AIC: Akaike information criterion

BSA: Bovine serum albumin

CI: 95% confidence interval

CV: Coefficient of variance

EIA: Enzyme immunoassay

FAM: Faecal androgen metabolites

FCM: Faecal cortisol metabolites

FSH: Follicle stimulation hormone

h^2: Heritability

LH: Luteinising hormone

LOD: Limit of detection

LRT: Likelihood ratio test

MRS: Maternal reproductive status

NNR: National Nature Reserve

SE: Standard error

SNH: Scottish Natural Heritage

V_A: Additive genetic variance

1 | General Background

Populations are comprised of individuals that differ from one another in their physiology, morphology, behaviour, life history and environmental experiences. Understanding this combination of intrinsic and extrinsic drivers of variation, and its fitness consequences, is crucial to understanding the ecological and evolutionary potential of populations, and through that, species. Considering the role of physiological variation, hormone concentrations differ markedly between individuals at all stages of life (Williams 2008), however little is known about the causes of this variation or its implications for wild populations. This thesis takes a multi-faceted approach to address this paucity of information within a single population of wild red deer (*Cervus elaphus*) on the Isle of Rum National Nature Reserve (NNR) in Scotland. The following chapters explore the potential for intrinsic and extrinsic drivers to account for inter-individual variation in the levels of two key hormone groups, androgens and glucocorticoids at different stages of life, and the implications of this variation for reproductive fitness and survival. In this introductory chapter, I provide general background to the area via a review of the relevant literature, dealing in turn with the biochemistry of hormones in animals, and then more specifically the role of steroids in fitness and life history trait expression. I then discuss past research on the causes of steroid hormone variation between individuals, and review the different methods of measuring this hormone variation. This introductory chapter finishes with an overview of my study system, a wild red deer population on the Isle of Rum NNR, and a brief outline of the general aims and questions of this thesis.

1.1. Hormone biochemistry in animals

Hormones are the chemical messengers of the endocrine system. These bio-regulatory molecules are synthesised by glands or specialised cells and secreted into the bloodstream (Becker et al. 2002a). This provides a sustained response to stimuli which complements the more rapid, short-term response of the nervous system (Squires 2003). Together, these two response systems make up the neuroendocrine system which is vital in the regulation and maintenance of body homeostasis (Becker et al. 2002a). The timing and quantity of hormone secretion is determined by a complex neuroendocrine pathway beginning in the hypothalamus, and controlled

by a number of feedback loops (Figure 1.1). Once in the bloodstream, hormone molecules are circulated around the body until they form complexes with hormone-specific receptors, usually on the surface of the target cell. This initiates a biochemical cascade effect (Hiller-Sturmhofel 1998), resulting in changes to the function or behaviour of that cell, and ultimately leading to physiological or behavioural responses in the organism.

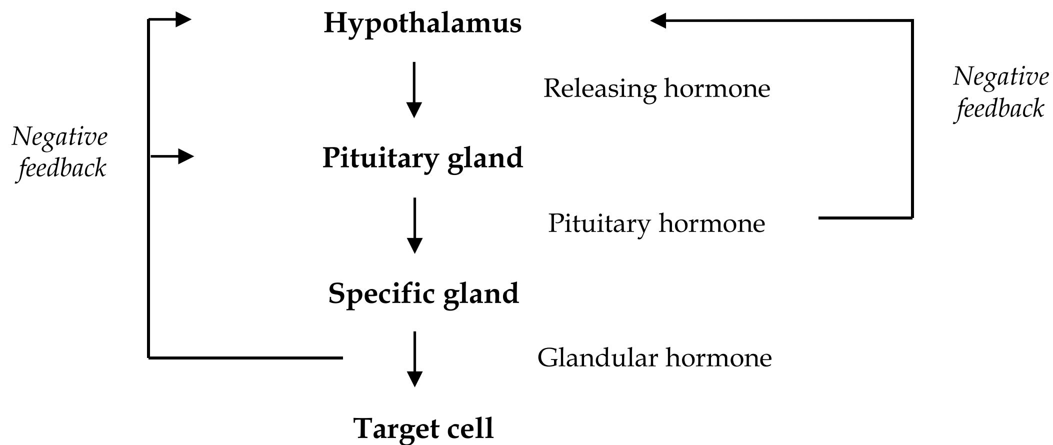


Figure 1.1*: Overview of a standard hormone biochemical cascade. The hypothalamus initiates a pathway of hormone secretions which result in change to the target cell.*

1.1.1. Steroid hormones

Steroids are a particular group of hormones which derive from cholesterol, and include both the gonadal and adrenal hormones. They are all similar in their molecular structure and are closely linked through the steroid biosynthesis pathway (Figure 1.2). All steroids are lipid soluble, allowing them to cross cell membranes to form hormone-receptor complexes intra-cellularly (Evans 1988). From here they move into the cell nucleus where they affect their target genes directly (see Squires 2003 for detailed discussion of the mechanisms involved).

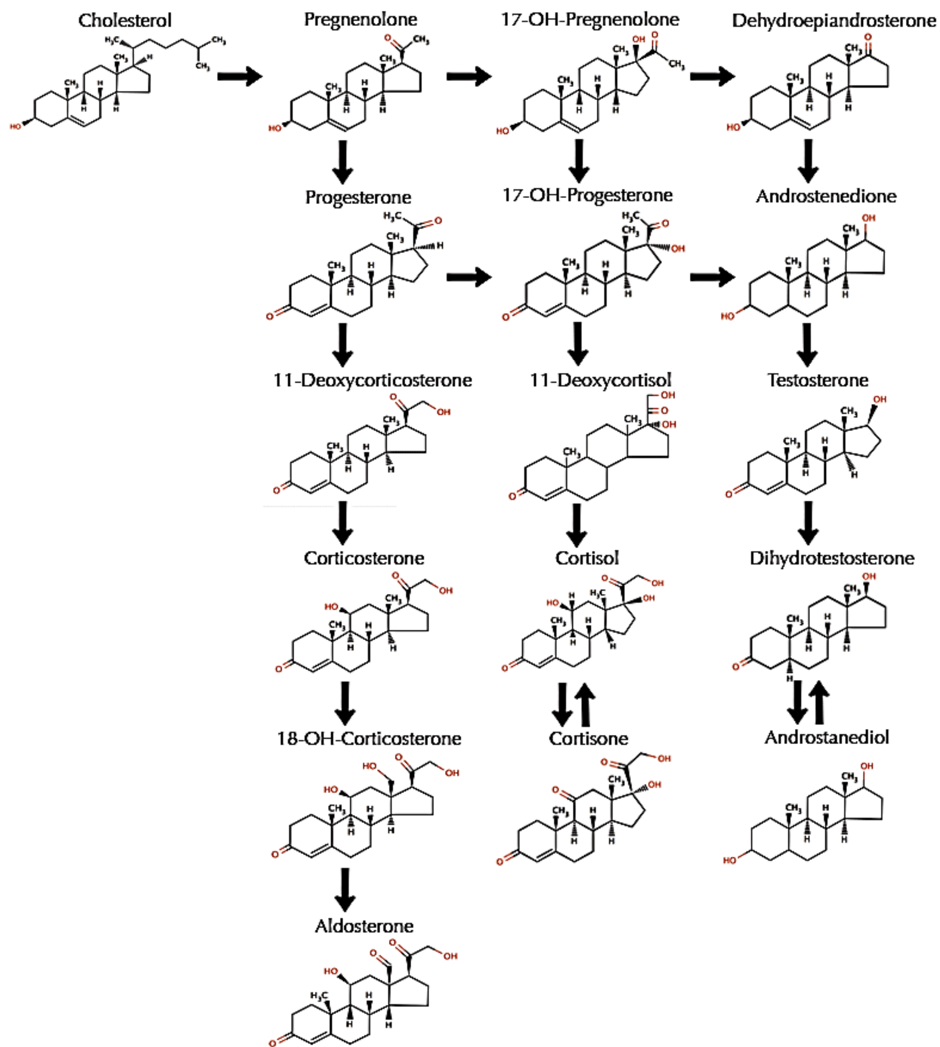


Figure 1.2: *Steroid biosynthesis pathway (credit: Tulane/Xavier Center for Bioenvironmental Research http://e.hormone.tulane.edu).*

The gonadal hormones (androgens, estrogens and progestagens) are synthesised primarily in the gonads, and are involved in the functioning, development and regulation of reproductive organs and secondary sexual characteristics. The adrenal steroids (mineralocorticoids and glucocorticoids) are produced in the cortex of the adrenal glands, and are important in homeostasis and the stress response (see Table 1.1 for a more detailed list of functions). This thesis concentrates on two of these hormone groups: the androgens (including testosterone) and the glucocorticoids (of which cortisol is the main one found in deer).

Table 1.1: *Main classes of steroid hormones, sites of production and function (Stocco and Clark 1996, Becker et al. 2002a, Squires 2003).*

Class	Examples	Primary site of production	Function
Androgens	Androstendione Dihydro-testosterone Testosterone	Testes (Leydig cell); Adrenal gland (cortex); Also produced in small quantities by the ovaries	Development of sexual features & behaviour (♂); Gender determination; Metabolism; Muscle & bone development; Spermatogenesis
Estrogens	Estradiol Estriol Estrone	Ovaries; Also produced in small quantities by the testes	Development of sexual features (♀); Gender determination; Hypothalamus modulation; LH and FSH regulation
Progestagens	Progesterone	Ovaries	Lordosis; Maintain pregnancy; Uterus preparation for pregnancy;
Gluco-corticoids	Cortisol Corticosterone	Adrenal gland (cortex)	Fat & protein degradation; Mediate stress response
Mineralo-corticoids	Aldosterone	Adrenal gland (cortex)	Salt balance; Water balance

1.1.2. Steroid metabolism

Steroid hormones circulate the body in their "native" form but are usually metabolised by the liver before being excreted in the urine and faeces (Mostl and Palme 2002). Steroids metabolised in the liver may either be excreted in the urine, or enter the gut where they are further metabolised by gut bacteria before excretion in the faeces (Mostl and Palme 2002). The relative proportion of metabolites excreted in the faeces versus the urine varies enormously between species; for example 82% of cortisol metabolites are excreted via the faeces in cats (Schatz and Palme 2001), whilst only 8% are excreted in the faeces in hares (Teskey-Gerstl et al. 2000). There are also species differences in the metabolites produced due to inter-specific differences in liver enzymes and gut biota (Palme 2005, Goymann 2012). Because of the large number of metabolites which are usually produced (Palme et al. 2005), they tend to be grouped according to particular molecular substructures. For

example, the assay validation I present in chapter 2.5 tests for the presence of three groups of androgen metabolites which have either 17α-OH, 17β-OH or 17-oxo groups as part of their molecular structures.

1.2. Steroids & trait expression

Steroid hormones are known to play important roles in the expression of a range physiological, morphological and behaviour traits, many of which have direct links to fitness. For example, male testosterone levels are associated with the initiation of aggression, courtship behaviour, and the development of ornamentation (both for display and weaponry) (Zuk et al. 1995, Lank et al. 1999, Book et al. 2001). Oestrogens are important in physiologically preparing the female body for pregnancy, and initiating maternal behaviours (Rosenblatt et al. 1994). Lordosis, a behaviour indicating female receptivity, is triggered by a surge of progesterone (Becker et al. 2002b). When levels of circulating glucocorticoids increase in response to stressors, this leads to a suite of physiological and behavioural changes which better equip the individual to rapidly respond to that stressor (e.g. Wingfield 2003). Whilst hormones are associated with the initiation and maintenance of trait expression across species, it is also important to understand how hormone levels differ between individuals, and what this can tell us about individual differences in hormone-linked traits (Williams 2008).

1.2.1. Effects on individual fitness-related traits

Whilst the importance of understanding the role of individual hormone variation is well recognised (e.g. Kempenaers et al. 2008, Williams 2008), it remains an understudied area, particularly when considering natural levels of phenotypic variation observed in wild populations. The experimental manipulation of individuals' hormone levels within a population has been used to illustrate the relationships between steroids and trait expression across a range of systems (e.g. Ketterson et al. 1991, Dachir et al. 1993, Lank et al. 1999, Buchanan et al. 2001, Jawor et al. 2006, Reed et al. 2006, O'Connor et al. 2009, Dey et al. 2010, Parker and Mason 2012), however this method only draws comparisons between two extreme groups of individuals. Whilst this is useful in illustrating fitness consequences of

physiologically high hormone concentrations, it still fails to consider the implications of more subtle natural inter-individual variation. In measuring natural circulating levels of estradiol in female late-run sockeye salmon (*Oncorhynchus nerka*) for example, Cooke et al. (2006) shows higher concentrations to be linked to a shorter holding phase and increased mortality during the spawning migration. In wild red deer, high testosterone has been linked to higher sperm quality and antler strength (Malo et al. 2009), and in superb fairy-wrens (*Malurus cyaneus*) an individual male's testosterone concentrations are positively related to their social dominance (Peters et al. 2001).

Measuring naturally-occurring individual variation is also important for understanding trade-offs between different characteristics (e.g. Mason et al. 1993, Mills et al. 2009). For example, testosterone has been linked to suppression of the immune system in high concentrations (Owen-Ashley et al. 2004), leading to elevated parasite load (Malo et al. 2009) and a proposed trade-off between testosterone-mediated sexual characteristics and immune costs (e.g. Schroderus et al. 2010). This led to the hypothesis that only the fittest males can cope with the costs incurred from maximising secondary sexual traits (Folstad and Karter 1992) and can therefore be used as an honest signal of male health or "quality" (Olsson et al. 2000, Mougeot et al. 2007). Whilst the stress response is important in reassigning resources during periods of acute stress, chronically high levels of glucocorticoids are also known to suppress both the immune (Romero and Butler 2007) and reproductive (Liptrap 1993, Dobson and Smith 1995) systems, as well as potentially lead to cellular damage (Sapolsky et al. 1986). The Cort-Fitness Hypothesis (reviewed in Bonier et al. 2009a) predicts that individuals with higher baseline levels of glucocorticoids will have lower overall fitness due to these immune and reproductive costs. As with the theory of testosterone-linked fitness trade-offs, however, this hypothesis remains poorly understood in wild and un-manipulated populations (though see Escribano-Avila et al. 2013).

1.2.2. Testosterone & sexual antagonism

Whilst many steroids differ between the sexes, arguably one of the best studied is testosterone, where high concentrations appear to have different implications for male and female performance. Research in birds suggests that selection on circulating concentrations acts primarily on males (Ketterson et al. 2005, Moller et al. 2005), however, in a hormone so integral to sexual differentiation it cannot be assumed that fitness benefits will be consistent across both sexes. Indeed, the potential for testosterone-linked sexually antagonistic selection has been suggested for a number of species, including mammals (Mokkonen et al. 2012), birds (Ketterson et al. 2005, Muller et al. 2005, Saino et al. 2006, Gerlach and Ketterson 2013, Rosvall 2013), and lizards (Hews and Moore 1995). In a captive study population of bank voles (*Myodes glareolus*), for example, male testosterone levels are shown to be heritable (Schroderus et al. 2010), positively correlated with male fitness (Mills et al. 2012, Mokkonen et al. 2012), and negatively correlated with immunocompetence in both sexes (Schroderus et al. 2010). Whilst there is the potential for higher testosterone levels to evolve in males, this is likely to be constrained by the net negative effects of high testosterone in females. Population-level experimental studies in birds also show these sex-differences: barn swallow (*Hirundo rustica*) eggs injected with testosterone result in smaller female and larger male chicks that their siblings from un-manipulated control groups (Saino et al. 2006).

Most studies agree that when there is a common genetic architecture in both sexes, the benefits of high testosterone in males, and the potential costs to females will prevent either sex from reaching their fitness optima. In the dark-eyed junco (*Junco hyemalis*), Clotfelter et al. (2004) demonstrate how females with relatively high testosterone may incur costs such as decreased body mass and delayed reproduction. They also suffer similar issues to their male counterparts with high testosterone, such as increased corticosterone and immunosuppression (Zysling et al. 2006). Whilst the fitness and reproductive benefits of elevated testosterone may outweigh the costs in males (e.g. through an increase in extra-pair copulation, Reed et al. 2006), no such benefits have been identified in females of the species. If a

genetic correlation exists between male and female testosterone concentrations, then the negative female selection may act as a constraint on increasing male fitness through elevated testosterone. Whilst there is some evidence in the literature inferring a cross-sex genetic correlation for testosterone concentration (e.g. Ketterson et al. 2005, Moller et al. 2005, Mank 2007, Schroderus et al. 2010), prior to analyses in the following chapters this had only been quantified once in human twins (Hoekstra et al. 2006). I explore these issues in more depth in chapter 4 where I directly test for the presence of a cross-sex genetic correlation in neonatal calf testosterone levels.

1.3. Causes of hormone variation

Individual variation in hormone concentrations is much more than measurement error around a population mean. Understanding the potential drivers of this variation at the individual level is crucial to fully answer questions about a population's ecology and ability to adapt to changing conditions. Hormone concentrations vary within and between individuals due to both intrinsic (e.g. genetics, maternal effects, and age) and extrinsic (e.g. social interactions and climate) drivers. Whilst an individual's hormone levels at a given time will result from a combination of these factors, previous studies have predominantly focussed on extrinsic factors, and they have rarely been examined together within a single study system. Below, I consider both intrinsic (genetics and maternal effects) and extrinsic (season/climate and social environment) drivers of variation, and discuss how they might cause hormone concentrations to differ between individuals.

1.3.1. Genetic variation

Phenotypic variation between individuals is the result of a combination of genetic and environmental influences (Falconer and Mackay 1996, Lynch and Walsh 1998). The heritability (h^2) of a trait provides a measure of the degree to which a trait is genetically determined, and has been shown to account for inter-individual hormone variation in a range of species (see Table 4.1 for a comprehensive list of testosterone heritability studies). Understanding how much an individual's

hormone concentrations are determined by their genes is important in ascertaining the potential for selection pressures to change mean population levels.

To date, most studies of hormone heritability have focussed on either human paired-relative studies (Meikle et al. 1988, Ring et al. 2005) or captive and laboratory populations (e.g. Evans et al. 2006, Schroderus et al. 2010; see also Table 4.1), which cannot be considered representative of natural populations (see Calisi and Bentley 2009 for further discussion). Little is currently known about the heritability of hormone concentrations in the wild (though see King et al. 2004), probably owing to the difficulty in obtaining sufficient and reliable data. Chapter 4 targets this current lack of information, investigating the heritability of plasma testosterone levels in neonatal red deer calves. Where heritability data are available, estimates appear hugely variable with both age (Ring et al. 2005) and population (Table 4.1), no doubt reflecting the fact that they are population-specific (Falconer and Mackay 1996). As well as explaining variance in absolute hormone titre measures, individual variation in the plasticity of hormone concentrations (e.g. the rate or extent of hormone response to stimuli) may also be explained by genetic variance (Mackinnon et al. 1991). The responsiveness of glucocorticoid levels to stressors, for example, has been shown to have a genetic component (Pottinger and Carrick 1999, Evans et al. 2006), although this is difficult to study in the wild because it requires regular and repeat sampling of individuals before and after a stressor.

1.3.2. Maternal effects

An individual's phenotype can be heavily influenced by their maternal environment during development, with effects extending far beyond the age when offspring would be directly affected by the presence of their mother (Wolf and Wade 2009). Maternal effects are distinguishable from the genetic effects discussed above in that they concern the non-genetic effects of the maternal environment on the phenotype of the offspring (Falconer and Mackay 1996, Wolf and Wade 2009). Maternal effects vary between individuals due to differences in maternal traits and condition. To date, the study of maternal steroid effects has been dominated by avian systems, both because birds develop outside the mother's body, and because females are

known to transfer a large (and variable) amount of steroids into their eggs (Schwabl 1993, Williams et al. 2005, Groothuis and Schwabl 2008). This allows the manipulation of egg hormone concentrations, mimicking variation in maternal hormone transfer, and subsequent investigation into the implications of this for offspring phenotype and fitness. For example, injecting house sparrow (*Passer domesticus*) eggs with testosterone has led to the development of males with larger badges, a positive fitness-related trait (Strasser and Schwabl 2004). Maternal hormone deposition may also show high within-brood variation, although this appears to be linked to maternal quality (Sasvari et al. 1999, 2006). For example, in tawny owls (*Strix aluco*), mean brood testosterone does not differ between high and low quality females, however the allocation of within-brood testosterone does (Sasvari et al. 2006). Whilst broods from high quality females show only marginal declines in testosterone with subsequent chicks, the negative relationship between chick testosterone and hatching order is highly significant in low quality females, irrespective of whether the chicks were given supplementary feeding.

Compared to avian work, we know relatively little about the role of maternal effects in determining hormonal variation in mammals. Whilst experimental hormone manipulation has been used in mammals, the additional presence of the placenta means that the relationship between maternal and foetal hormones is less well understood than in birds. There is evidence that several key hormones, including testosterone, do not pass across the placenta in many mammal species: for example, guinea pigs (*Cavia porcellus*) (Vreeburg et al. 1981, Despres et al. 1984) and rats (Slob et al. 1983, Sathishkumar et al. 2011, Sun et al. 2012), though this is not true for hyenas (*Crocuta crocuta*) (Licht et al. 1998). Furthermore, the placenta synthesises its own hormones (Strauss et al. 1996, Hoffmann and Schuler 2002, Khatri 2011, Mondragon et al. 2012). Nevertheless, despite not fully understanding the mechanisms, elevated maternal testosterone in the peripheral blood has been linked to the masculinisation of developing female foetuses in a number of species (e.g. Clarke et al. 1976 and references therein). Stress during pregnancy is also linked to hormone-mediated effects on (often sex-specific) offspring trait expression and fitness (Kaiser and Sachser 2005). Variation in maternal condition has also been linked to variation in offspring hormone concentrations (Ward et al. 2002, Sasvari et

al. 2006, Von Engelhardt and Groothuis 2011), although this area of research is poorly represented in the literature. Chapters 3 & 6 address these issues by examining the role of maternal traits in explaining an offspring's neonatal testosterone levels.

1.3.3. Seasonal and climatic variation

Hormone concentrations are also influenced by extrinsic factors, such as climatic variation. Hormones can be influenced by weather in different ways, and different individuals may respond differently to the same conditions (e.g. Saltz and White 1991). A number of studies show hormones to exhibit seasonal cycles (e.g. Wingfield and Farner 1980, Suttie et al. 1989, Romero 2002), often with close correlations with the reproductive cycle of the species (Malpaux 2006). For example, male testosterone may increase when females become sexually receptive (Runfeldt and Wingfield 1985, Wingfield et al. 1990).

Whilst many of these seasonal changes have been linked to photoperiod (e.g. Barrell et al. 2000, Malpaux 2006), endogenous hormone cycles are also shown to persist in experimental animals kept at constant photoperiod, although not necessarily to a circannual rhythm (e.g. LH in sheep: Karsch et al. 1989). In addition to these more predictable cycles, extreme or atypical weather events may also influence an individual's hormone levels. For example, testosterone and LH concentrations in free living white-crowned sparrows (*Zonotrichia leucophrys pugetensis*) have been shown to remain elevated for longer during the breeding season in the presence of atypical spring storms . Weather may also influence an individual's hormone levels indirectly, for example by reducing condition (Cook et al. 2002) and increasing stress (Saltz and White 1991, Huber et al. 2003a) during bad weather. Understanding these transient changes due to extrinsic effects is important in better understanding how sensitive hormone concentrations (and their resulting effect on hormone-mediated traits) will be to environmental instability, the threat of extreme weather, and change from previous climatic norms (e.g. in fish: Pankhurst and Munday 2011). In the following chapters, seasonal cycles in concentrations of faecal androgen and cortisol metabolites are examined in both males (chapter 5) and

females (chapter 6) along with other extrinsic and intrinsic factors. Appendix B also explores the effects of weather during foetal development on neonatal testosterone levels in calves.

1.3.4. Social environment

Finally, in common with many areas of evolutionary ecology, there is increasing appreciation of the inter-dependence of an individual's phenotype and their social environment. Numerous behavioural traits are known to be influenced by relative hormone levels, including dominance and aggression (Book et al. 2001, Muller and Wrangham 2004a, Summers et al. 2005). The reverse can also be true, with studies from a range of taxa including birds (Wingfield et al. 1990, Hirschenhauser et al. 2003), mammals (Bowman et al. 1978, Shargal et al. 2008) and fish (Elofsson et al. 2000) showing hormone levels to be influenced by an animal's social interactions. These hormone responses to social cues tend to be transient in their nature, with changes in social condition or state leading to changes in measurable hormone concentrations within individuals (Oliveira 2004). For example, glucocorticoid concentrations increase following a stressful encounter (Sapolsky et al. 2000, Greenberg et al. 2002), and a male's testosterone levels will often increase as they enter the breeding season, often linked to the acquisition and defence of mates and/or territory (Wingfield et al. 2001, Lynch et al. 2002). The degree to which an individual's hormone levels vary from their conspecifics', both in absolute concentration and the degree to which they respond to social stimuli, however, is hugely variable (Kempenaers et al. 2008). This may reflect individual variation in life history strategy, for example androgen concentrations are linked to trade-offs between survival and reproduction, and between or male mate acquisition and paternal care (Oliveira 2004, Pinxten et al. 2007).

As well as varying in their response to social interactions, hormones can also differ between individuals as a consequence of differences in social status, although not always predictably so. Social dominance is often assumed to be directly linked to high testosterone, however studies are increasingly showing that this is not always the case (Sapolsky 1992, Mason et al. 1993, Bartos et al. 2010). Relationships between

an individual's hormone levels and their dominance status appear to be determined by the social stability of their dominance hierarchy. Higher testosterone and lower glucocorticoids are often only associated with more dominant individuals when they are in stable hierarchies. When hierarchies become unstable (or exist in a unstable state) higher ranking individuals are often found to have *lower* testosterone and *higher* glucocorticoids than their conspecifics, presumably because of multiple, evenly-matched individuals competing for dominance (Sapolsky 1992, Mason et al. 1993, Bartos et al. 2010). An individual's hormone concentrations, therefore, are not only related to their own inherent state but also to the behaviour of, and interactions with, surrounding individuals. This is clearly illustrated by changes to an individual's hormone concentrations as a consequence of witnessing (but not being involved in) aggressive interactions (e.g. Oliveira et al. 2001). As with the effect of climatic variation, different individuals may exhibit different hormonal responses to social or behavioural stimuli, and it is these differences which best enable us to understand how hormones may mediate links between external environmental (be they climatic or social) and consequential physiological or fitness responses.

1.4. Measuring hormone concentrations

The previous sections considered sources of variation in hormone concentrations, whilst here I discuss how we might go about investigating them. Hormone concentrations can be obtained and measured from a range of different biological samples including: blood plasma & serum, faeces (Schwarzenberger 2007), urine (e.g. Hay and Mormede 1998), saliva (Groschl 2008), milk (e.g. Verkerk et al. 1998), hair (e.g. Koren et al. 2002), feathers (e.g. Bortolotti et al. 2009) and antler (e.g. Bubenik et al. 2005). At present, most analyses utilise hormone measures originating either from blood or faecal samples, the pros and cons of which are summarised in Table 1.2. Traditionally, hormone studies have focussed almost exclusively on blood samples because they are easy to obtain experimentally and provide the most direct measure of immediate circulating concentrations (Becker et al. 2002a). Whilst blood sampling is useful in many situations, it does have some inherent problems (see Table 1.2). Firstly, many hormones are not secreted into the blood at a constant rate, rather they are released in pulses (e.g. Karsch 1987, Young et al. 2004), meaning that

hormone concentrations can vary substantially across a very small window of time. Secondly, sampling blood is an invasive procedure which will likely initiate stress responses in the sampled individuals. Glucocorticoids can increase in the blood within 3 minutes of a stressor (Romero and Reed 2005), making it difficult to obtain baseline (or even comparable) concentrations of these hormones from study organisms (see appendix A for analysis in this study population). This is particularly true for wild populations where endocrine studies could be valuable to conservation work. Invasively sampling blood is also more challenging, particularly when dealing with wild animals that need to be caught before sampling.

***Table 1.2**: Comparison between the two predominant types of biological sample from which hormone concentrations can be measured.*

	Blood	**Faeces**
Advantages	Identifiable individuals Direct measure of circulating hormone concentrations (Brown 1994) Control quantity of blood extracted Use of commercial assay kits Measures native hormone	Non-invasive/logistically easier for wild animals Allows baseline concentrations of stress hormones to be measured Can be frozen without processing (Millspaugh and Washburn 2004) Provides a mean concentration across several hours (Good et al. 2003) Doesn't effect "natural" behaviour
Limitations	Rapid stress response (<3 min; Romero and Reed 2005), makes baseline levels of stress hormones difficult to measure Invasive sampling which requires training in animal handling procedures to avoid damage and minimise stress Cannot repeatedly sample small animals because insufficient volume of blood available (Chelini et al. 2005) Hormones do not store well in blood (Tworoger and Hankinson 2006), so requires rapid plasma extraction: may be challenging in the field Pulsatile secretions mean that short term measures might not be repeatable within individuals	Hormones present in metabolised form (Palme 2005, Goymann et al. 2006) Individual identification can be challenging in some species (e.g. communal latrines) Concentrations (Goymann et al. 2006) or absolute amounts (Rabiee et al. 2001) of hormone may be affected by faecal mass Requires extensive immunoassay validation for each species (Touma and Palme 2005) Inter-individual variation may also arise due to differences in gut bacteria causing variation in excreted metabolites (Klasing 2005) Concentrations may be affected by dietary intake (Rabiee et al. 2001)

Due to the invasiveness of blood sampling, the use of non-invasive hormone sampling techniques has become an increasingly popular alternative. Amongst these, faecal sampling has had the widest uptake because whilst urine can be used

when collecting from trained animals, it is generally more difficult to obtain (Schwarzenberger et al. 1996).

Whilst blood and faecal hormone measures often correlate (see Schwarzenberger 2007 for discussion and references therein), this is not ubiquitous, nor does it undermine faecal hormone analyses, because these two hormone sources are not measuring the same thing (see Schwarzenberger et al. 1996, Palme 2005, Touma and Palme 2005 for discussion). Blood samples provide a snapshot measure of circulating hormone levels, whilst hormones measured in faecal samples are usually the metabolites of circulating hormones which are metabolised by gut micro-organisms prior to excretion (Palme 2005). Whilst levels of hormones measured in the blood provide a short-term measure of concentrations at the point of sampling, levels of hormone (and hormone metabolites) measured in faecal samples represent an aggregate of circulating levels across several hours.

The exact faecal metabolites of a native hormone vary between species due to variable gut fauna (Goymann 2012), therefore species-specific validation of immunoassays is required to ensure that they are biologically relevant (Touma and Palme 2005, Goymann et al. 2006). Once validated, however, the analysis of faecal hormone metabolite levels has enabled a far greater use of endocrine information than could have been permitted by blood analysis alone. This method is particularly valuable for studying wild animals or endangered species, and for ongoing monitoring in zoos and captive breeding facilities (e.g. Morrow and Monfort 1998, Walker et al. 2002, Terio et al. 2004, Schwarzenberger 2007) where invasive monitoring of individuals is often not possible.

Faecal sampling is not without problems (see Table 1.2), and there is still the risk that variation in concentrations may partly be an artefact of the conditions under which an individual is placed (see Millspaugh and Washburn 2004, Goymann 2012 for further discussion). Despite this, faecal analysis can be a powerful means of ascertaining hormone concentrations without the need for invasive procedures, and many effects can be identified and minimised by understanding the wider biology of the study system. Where this is not possible, or samples are not available, plasma

sampling remains an important method, but this comes with many caveats that need to be understood and accounted for when interpreting results. In this thesis I use hormones collected from both blood plasma and faecal samples to explore individual differences in androgen and glucocorticoid levels.

1.5. Red deer on Rum

In this thesis, questions about individual-level hormone variation are explored within a wild population of Scottish red deer (*Cervus elaphus scoticus*) on the Isle of Rum NNR. The animals are descended from a population of red deer translocated to the island from mainland Scotland in 1845 to replace a previous population that went extinct (Clutton-Brock et al. 1982). Red deer have a widespread distribution (Figure 1.3) comprising of a number of highly variable subspecies. The Scottish red deer is a European sub-species notable for its diminutive size (Huxley 1932), which is likely to result from its relatively poor graze (Huxley 1932, Lowe 1961).

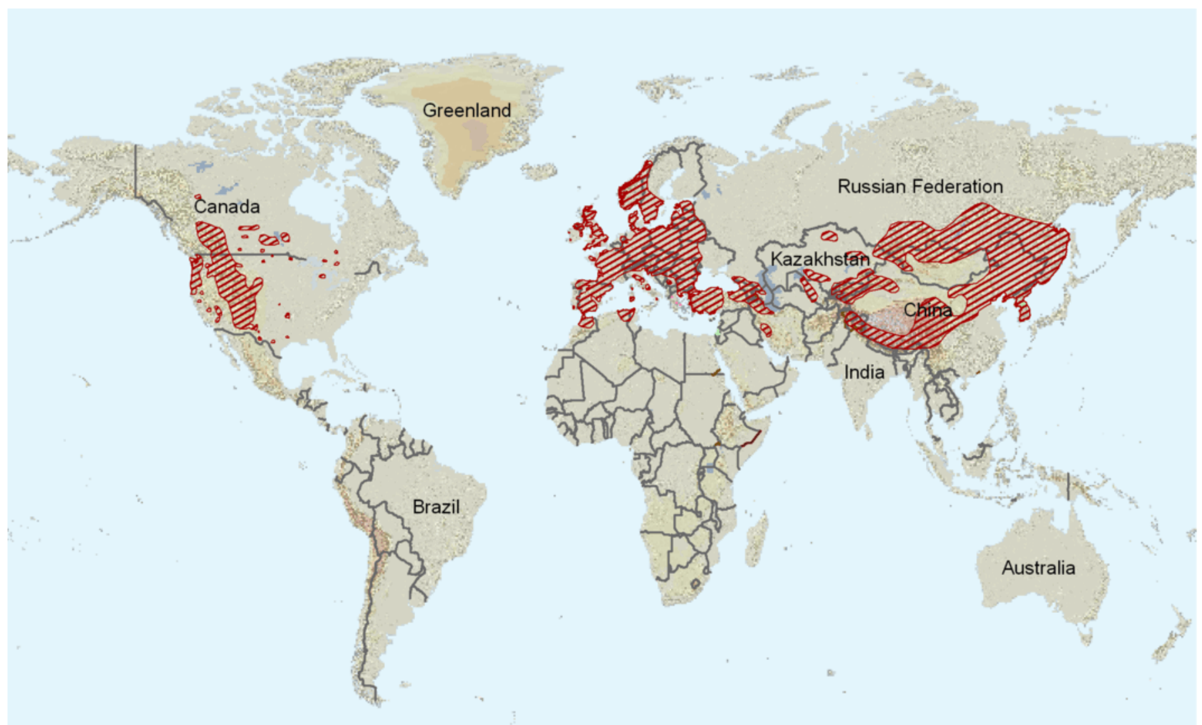


Figure 1.3: *Current distribution of native red deer populations reproduced from the IUCN Red List (Lovari et al. 2008). For the purpose of simplicity, the European red deer and wapiti (North American/Asian red deer) are treated as a single species.*

1.5.1. Species life history

Red deer are polygynous capital breeders (Moyes et al. 2006) which exhibit clear sexual dimorphism, and with the exception of the rut (mating season) there is little interaction between adult stags and hinds. On the Isle of Rum the rut falls between September and early November, peaking in mid-October (Figure 1.4), and is characterised by male-male competition for sexually mature females. At its peak, older and more experienced stags will monopolise access to females, whilst younger or subordinate stags will mate opportunistically or not at all (Clutton-Brock et al. 1979).

Females show strong site philopatry, and daughters often establish territories in the locality of their mother. Males, meanwhile, disperse from the natal herd and often form their own bachelor groups beyond the study area (Coulson et al. 1997). There is also a marked sexual differentiation in reproduction. Whilst most hinds will calve every one or two years from the age of three or four, male reproductive output is highly skewed (Foerster et al. 2007). A few stags will sire the majority of calves in a cohort, and many will never produce any offspring. Indeed, in an early study of 1971-1977 cohorts, Gibson & Guinness (1980) found that nearly half the study males failed to breed in any one year, whilst the most productive 5% in the year produced at least five offspring.

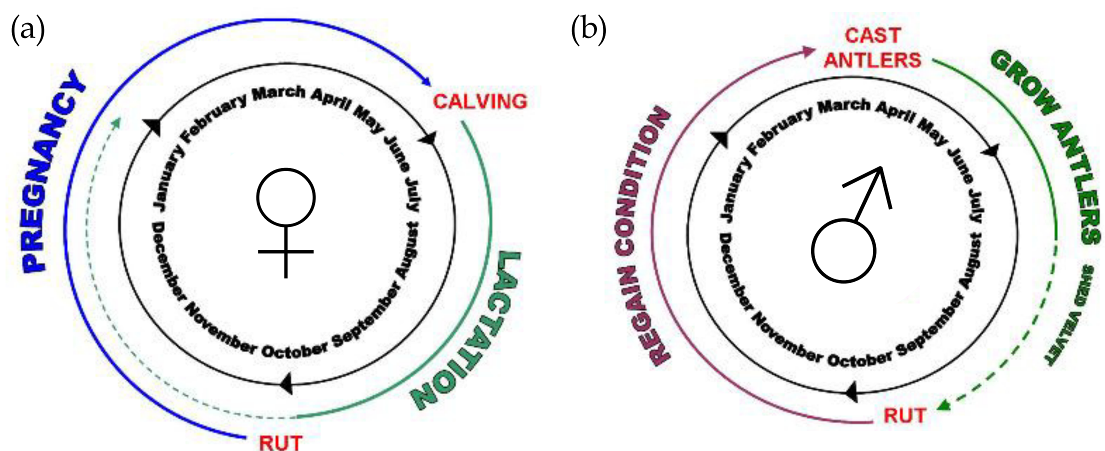


***Figure 1.4**: Generalised reproductive cycles for (a) hinds and (b) stags, reproduced from Nussey et al. (2009).*

1.5.2. Isle of Rum NNR

The Isle of Rum is a small Scottish island in the Inner Hebrides. It has an area just over 100 km^2, and is populated by approximately 1000 red deer. It is divided into five deer management blocks (Figure 1.5) drawn out by the Nature Conservancy Council (now Scottish Natural Heritage: SNH) following their purchase of the island in 1957. The biodiversity of Rum is considered both nationally and internationally important, and this is reflected in the island's numerous designations: including as a NNR, a Site of Special Scientific Interest, a Special Area for Conservation, and a Special Protected Area (SNH 2009). As the red deer on Rum have no natural predators on the island (with the except of golden eagles (*Aquila chrysaetos*) which occasionally predate calves), red deer grazing is considered a key problem in preventing the establishment of native woodland, one of the key goals laid out by SNH (SNH 2009).

Traditionally the deer were managed by uniform culling across the island, with around 14% of individuals removed annually (Clutton-Brock et al. 1982), however in 1972 the North Block (Block 4 in Figure 1.5) became exempt from this cull. Research on the deer in the North Block began in the 1960s, but it was not until the release from culling that a long term system of individual monitoring was introduced, along with regular censuses and a system for individually marking and identifying animals (Clutton-Brock et al. 1982).

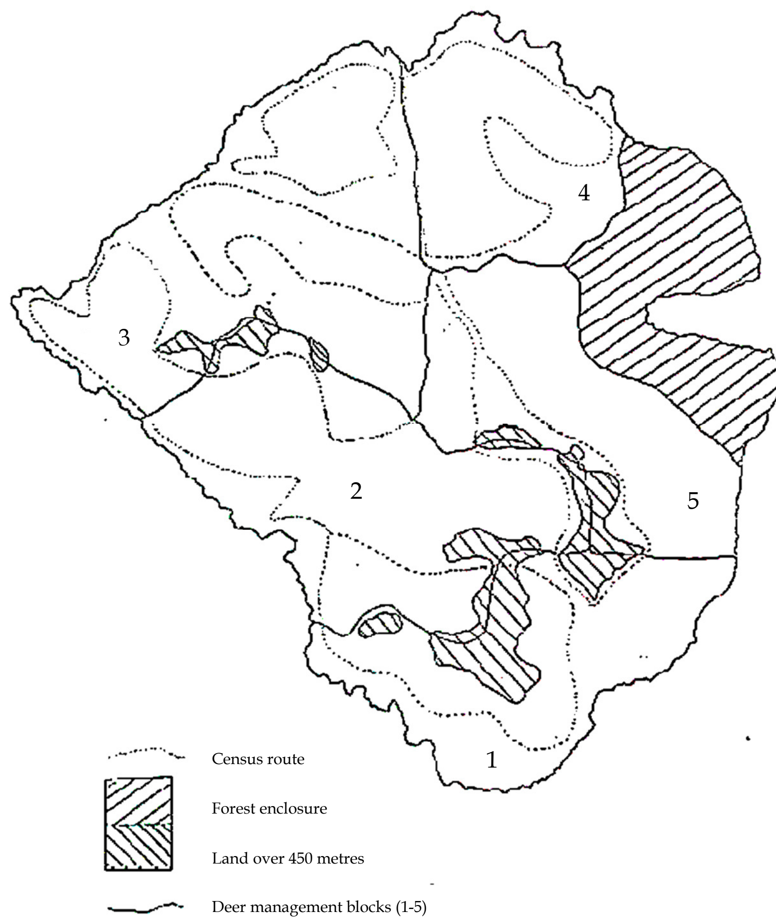


Figure 1.5*: The Isle of Rum NNR divided into its five management blocks (reproduced from Clutton-Brock, 2011). The North Block is labelled 4.*

1.5.3. Value of long-running study populations

Populations are not static units, but fluctuate extensively across time. Long-running data sets enable these dynamics to be followed, revealing patterns and relationships that may be undetectable across shorter time periods. By way of example, the long-running study population of feral Soay sheep on Hirta undergo crashes of up to 70% approximately every three years (Grenfell et al. 1992, Clutton-Brock and Pemberton 2004). A temporal subset of this data would fail to capture the population's dynamic nature, and different conclusions would be drawn depending on the subset of years considered. Despite this, the number of species for which wild long-term individual-based data exists is limited (see Clutton-Brock and Sheldon 2010 for examples).

The same is true for understanding the implications of anthropogenic pressures. For example, it was found to take about a decade for a population of red deer to reach natural carrying capacity following their release from culling (Coulson et al. 2004), something impossible to ascertain without on-going monitoring. This highlighted a number of interesting demographic changes which could have important implications for improving the management of other ungulate populations, such as changes to the sex ratio. Topically, long-term data sets are also becoming increasingly valued in attempts to predict species response and resilience to environment change and stochasticity due to climate change (Stopher et al. 2014).

Without these long-term data sets, such drivers of change and subsequent population responses could not be understood. It is for this reason that they are so important to the conservation of rare species, allowing proactive management through enhancing predictive power (e.g. Brook and Kikkawa 1998) and guiding decision making (e.g. Sinclair et al. 2007). They are also valued by evolutionary and theoretical ecologists for the role they can play in addressing ecological questions such as adaptation, evolution and demographic stochasticity.

1.5.4. Red deer data collection

Most individuals within the North Block are individually identifiable, either through artificial or natural markings. Starting in 1971, individuals born in the study area have been caught as calves and marked with a unique combination of ear punches, numbered ear tags and (for females) expandable collars (see Figure 1.6 for picture of a tagged calf). Although not all animals are found and caught as calves, even unmarked animals frequently have unique morphological features which are identifiable to trained field workers. In addition to this, stags can also be identified from their antler morphology which, with the exception of increasing size, often remains fairly consistent year on year. Of the calves which are tagged (~70% of those born in the study area), most are caught within the first couple of days of life. During processing, morphological measures (e.g. leg length, weight) and biological samples (e.g. ear tissue punch, blood) are also collected. With the exception of a few

animals darted across the years, the deer are not handled or interfered with once they have been caught as calves. Regular mortality searches, particularly during the late winter period, allow for post mortem measures and samples to be collected from the carcasses. Date of death is often estimable to within a week, further allowing for relatively accurate lifespans to be calculated.

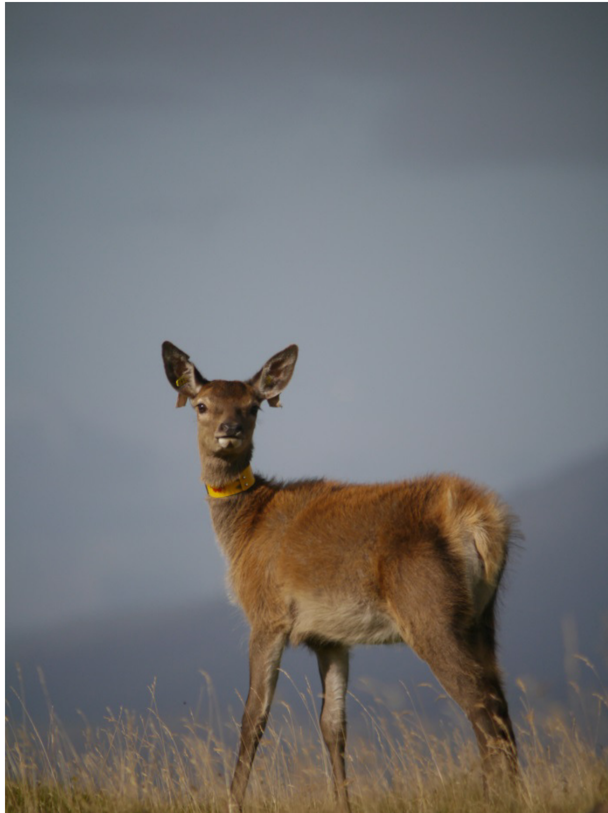


***Figure 1.6**: Female red deer calf (approx. 10-12 weeks old) with unique set of ear tags and expandable collar (photographer: A. Pavitt, 2012).*

A combination of regular censuses and the collection of biological tissues from calves, darted individuals, and carcasses enables the construction of a detailed pedigree for this population. Maternity is almost always assigned based on observations of mother and calf (Clutton-Brock et al. 1982), whilst paternity is based on a combination of rutting behaviour and genetic paternity analysis (Walling et al. 2010). Not only does this allow for heritability analyses to be conducted on this population (see chapter 4 for heritability estimates of neonatal testosterone), but also provides invaluable data on lifetime reproductive traits.

1.6. Thesis aims

In this thesis, I ask three major questions:
(a) How much do hormone levels vary between individuals in a wild population of Scottish red deer?
(b) What causes them to vary?
(c) Can this variation be used to better understand individual differences in fitness and life history traits?

I focus on two hormones: androgens (e.g. testosterone) and cortisol, the primary glucocorticoid in red deer. I also utilise two different sources of hormones: plasma and faecal samples (see chapter 1.4 and Table 1.2 for discussion of the differences between these two sources) which have been collected and stored as part of the long-running Rum red deer study. The long-term, individual-based monitoring of this study population provided the opportunity to ask a large suite of questions about natural hormone profiles within and across the same animals.

Laboratory methods, including details of sample collection, hormone extraction and assay methodology are detailed in chapter 2. This chapter also presents the first validation of an assay for faecal androgen metabolites (FAM) in red deer, which is subsequently utilised for both chapters 5 & 6.

In chapter 3 I consider individual differences in neonatal plasma testosterone concentrations in light of maternal quality and calf traits, as well as the implications of this variation for calf survival. This chapter forms the basis of Pavitt et al. (2014a). Chapter 4 builds on chapter 3, utilising animal models to explore the quantitative genetics of circulating neonatal testosterone levels, as well as the genetic correlation between male and female neonatal testosterone levels (see Pavitt et al. 2014b).

Chapters 5 and 6 are concerned with concentrations of FAM and faecal cortisol metabolites (FCM) across all ages in males (chapter 5) and females (chapter 6). Chapter 5 then considers age and season related variation in male FAM and FCM levels, and the relationship between a stag’s hormone concentrations and their

reproductive effort during the breeding season (Pavitt et al. In review). Chapter 6 explores the effect of age, season and reproductive status on FAM and FCM concentrations in females. After correcting for these causes of variation, the relationships between a female's hormone concentrations during pregnancy and the circulating testosterone levels in the resulting calf are then investigated.

Chapter 7 consolidates the findings of this thesis, discusses how they are inter-linked, and how they contribute to our understanding of steroid hormone concentrations within wild mammals.

2 | Methods

2.1. Sampling the Rum red deer

2.1.1. Blood collection

Circulating levels of neonatal testosterone were measured in the plasma of 920 individual red deer calves born between 1996 and 2012 in the North Block study area on the Isle of Rum NNR, Scotland (see Clutton-Brock et al. 1982 for full description of the study population and site). Female red deer give birth to a single calf, typically in May-June, and all samples were collected from calves aged 0-14 days old (mean age: 1.9 days ± 0.1 SE). Blood was collected into lithium heparin tubes when calves were caught for tagging, and other measurements including weight were also taken as part of an on-going programme of monitoring and research. Blood samples were centrifuged at 1500 g for 10 minutes and plasma removed and frozen within 24 hours of capture. To test whether capture affected testosterone levels, 18 calves were bled twice during the 2013 calving season, the first immediately after capture and the second 4-5 minutes later, which is when they were normally bled. All work was carried out in accordance with UK Home Office guidelines and regulations.

2.1.2. Faecal collection

A total of 1000 faecal samples were collected from individually identifiable red deer on the Isle of Rum NNR between the years 2001 and 2013. Of these samples, 194 were collected from males (n=73 different individuals), and 806 from females (n=200 different individuals), with many deer sampled more than once during this time period (Figure 2.1). All females were born in the study area, as were 53 of the 73 samples males. The remaining 20 males were born elsewhere on the island (consequently they were untagged but were identifiable from their antlers), and were sampled whilst visiting the study area (labelled 4 in Figure 1.5). Fresh faecal samples were collected both opportunistically and from targeted collection sessions within 5 minutes of witnessing defecation, and only from positively identified individuals. They were stored at -20ºC in a field freezer (mean time from collection to freezing: 101.6 minutes ± 10.1 SE), before being packed in ice and returned to laboratory freezers were they were kept at -20ºC until extraction.

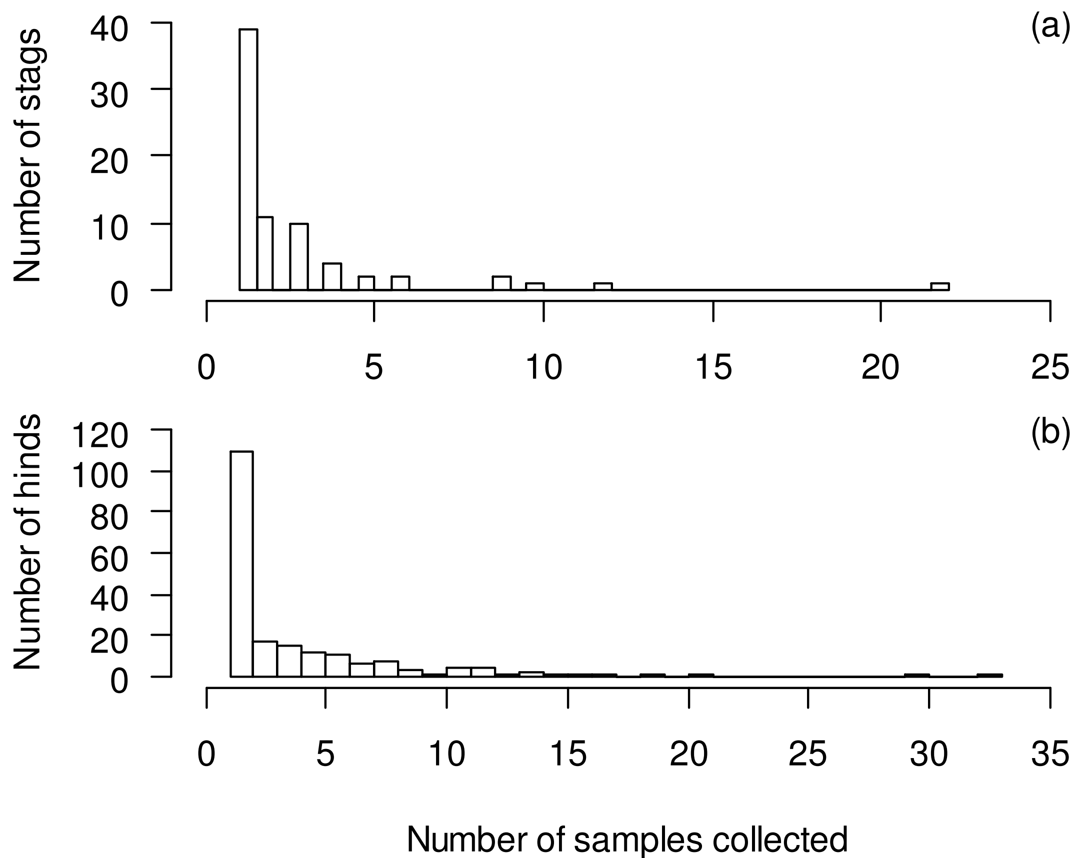


Figure 2.1: *Distribution of number of faecal samples collected per individual (a) male (n=194 samples; 73 males) and (b) females (n=812 samples; 200 females).*

2.2. Plasma hormone extraction

Hormones were extracted from 100 µl samples of blood plasma which were added to 2 ml of tert-butyl methyl ether and frozen in an ethanol/ dry ice bath. The ether layer was decanted into glass tubes, dried under nitrogen steam at 40ºC, and concentrated 2.5 times by reconstituting in 250 µl of re-suspension buffer PBS + 0.1% BSA (bovine serum albumin). Samples were stored at -20ºC until ready for use.

2.3. Plasma testosterone assay

Testosterone concentrations were measured using a commercially available testosterone competitive-binding enzyme immunoassay (EIA) kit (582701, Cayman Chemicals Ltd., USA), with all samples run in duplicate. The cross-reactivity was given as: 19-nortestosterone 140%, testosterone 100%, 5α-dihydro-testosterone (5α-

DHT) 27.4%, 5β-dihydro-testosterone (5β-DHT) 18.9%, methyl testosterone 4.7%, androstenedione 3.7%, 11-keto testosterone 2.2%, and <1% for all remaining compounds including progesterone (0.14%) and estradiol (<0.01%). Serial dilutions of pooled samples showed high parallelism with the standard curve ($p<0.001$) and no samples fell below the calculated limit of detection (LOD) (5.78 pg/ml). The intra- and inter-assay coefficients of variance (CV) were calculated at 5.13% and 11.66% respectively. Intra-assay CVs measure the similarity between duplicates of the same sample run on the same plate, providing a measure of pipetting accuracy, and are generally accepted if they fall <10%. Inter-assay CVs measure the similarity between standard controls replicated on all plates to test the precision and repeatability of the assay procedure across assay plates. These are usually acceptable <15%.

2.4. Faecal hormone extraction

Individual faecal samples were fully defrosted and homogenised to evenly distribute hormones throughout the faeces. Once homogenised, 0.5 g of wet sample was extracted with 5 ml of methanol (90%), gently shaken (overnight at 20ºC) and centrifuged (20 minutes at 652 g). Following this, 1 ml of the resulting supernatant was transferred to a clean tube and stored at -20ºC until assay.

2.5. FAM assay validation

Given that no FAM assay had previously been validated for red deer, I tested the ability of several androgen EIAs to detect biologically meaningful differences between a subset of faecal samples collected from red deer on the Isle of Rum NNR between 2006 and 2012. A total of 30 faecal samples (female: n=11; male: n=19) were collected from 25 wild red deer (female: n=9; male: n=16) to test the ability of three androgen EIAs to discriminate between sexes and male reproductive status: 17α-hydroxyandrogens (Mohle et al. 2002), 17β-hydroxyandrogens (Palme and Mostl 1994) and 17-oxo-androgens (Palme and Mostl 1994). All three of the tested assays were group specific and reacted with 4-ene and 5α-androstanes. The tested samples included 11 samples from females (age: 3-16 years old, mean: 8.7 years old ± 2.6 SE),

10 from males during the rutting season (age: 6-12 years old, mean: 7.9 years old ± 2.5 SE) and 9 from non-rutting season males (age: 4-12 years old, mean: 10.8 years old ± 2.6 SE). Samples from rutting season males were collected during September-October, and from non-rutting season males during February-April. Each of the tested assays was carried out following standard protocol (17β-hydroxyandrogens & 17-oxo-androgens: Palme and Mostl 1994, 17α-hydroxyandrogens: Mohle et al. 2002). The 17-oxo-androgen (epiandrosterone) assay detected the largest biological difference between sexes (t-test: p=0.005; Table 2.1; Figure 2.2), and between male rut and non-rut (t-test: p=0.001; Table 2.2; Figure 2.3) samples, and so was used in all subsequent analyses. 17-oxo-androgens are the faecal metabolites of 17β-hydroxyandrogens (including testosterone) and androstenedione (Palme and Mostl 1994), although this fraction may also include metabolites from adrenal androgens like dehydro-epiandrosterone (DHEA; Mohle et al. 2002).

Table 2.1: *Welch two-sample t-test results comparing female and male hormone metabolite concentrations measured using three different androgen assays.*

	t	**df**	***P***
17α-hydroxyandrogens	-1.601	19	0.126
17β-hydroxyandrogens	-3.023	19	0.007
17-oxo-androgens	**-3.155**	**19**	**0.005**

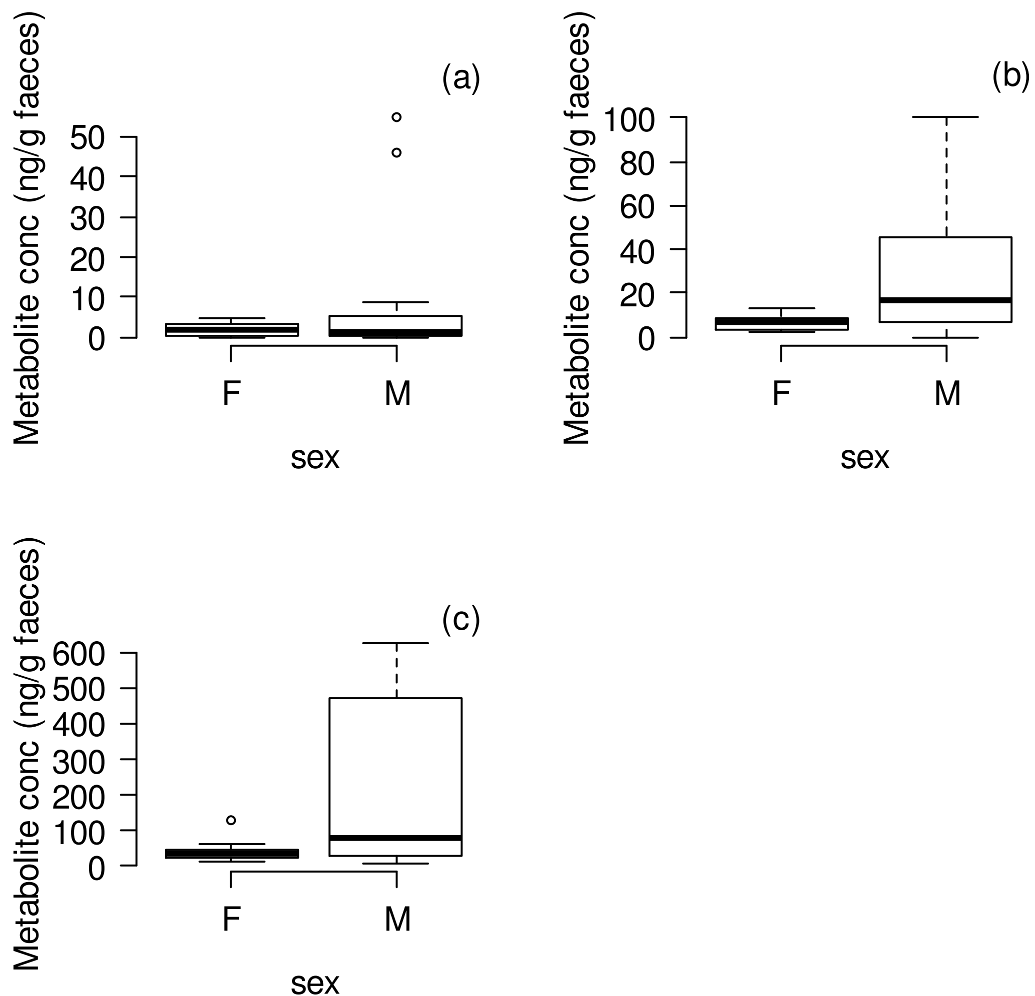


Figure 2.2: *Comparison between mean female (F) and male (M) hormone metabolite concentrations measured using (a) 17α-hydroxyandrogen, (b) 17β-hydroxyandrogen, and (c) 17-oxo-androgen assays.*

Table 2.2: *Welch two-sample t-test results comparing hormone metabolite concentrations from males during the rut versus non-rutting period measured using three different androgen assays.*

	t	df	*P*
17α-hydroxyandrogens	-2.168	9	0.058
17β-hydroxyandrogens	-3.988	9	0.003
17-oxo-androgens	**-4.639**	**9**	**0.001**

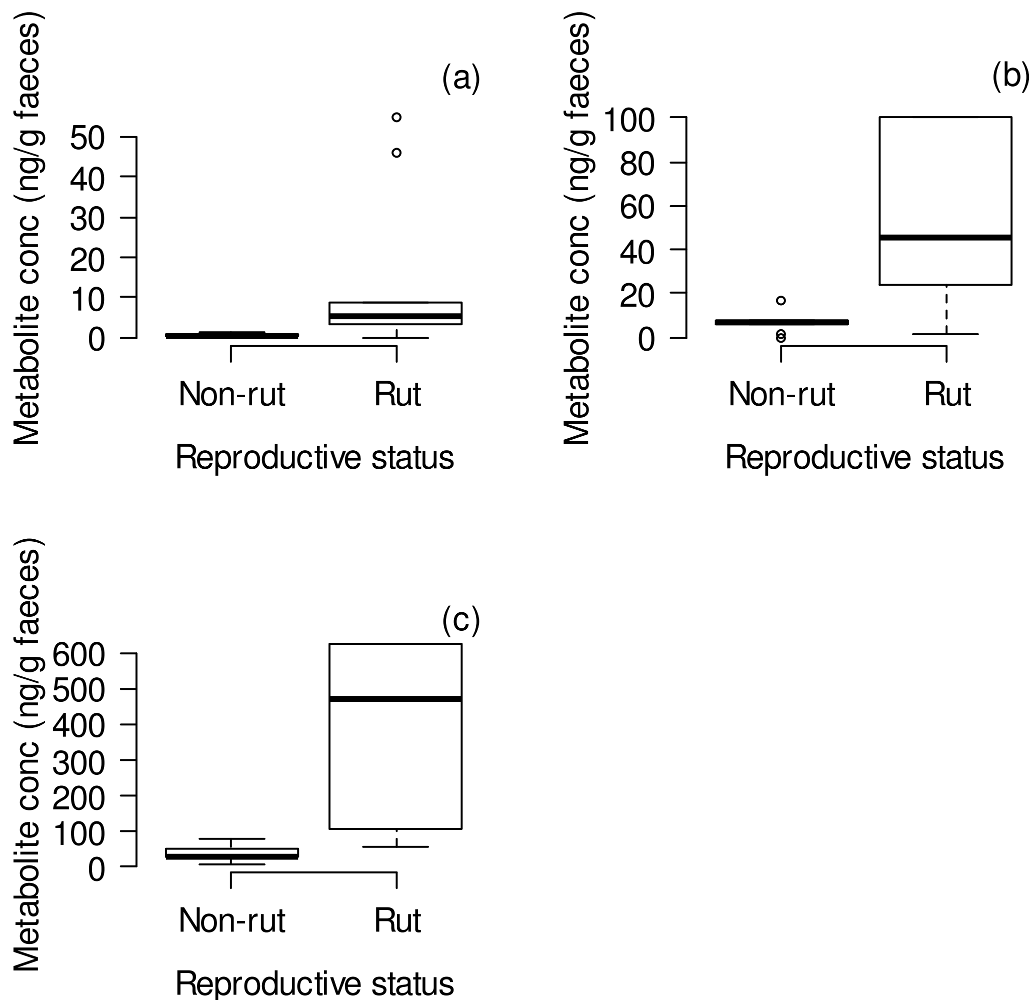


Figure 2.3: *Comparison between mean hormone metabolite concentrations from males during the rut versus non-rutting period measured using (a) 17a-hydroxyandrogen, (b) 17β-hydroxyandrogen, and (c) 17-oxo-androgen assays.*

2.6. FAM immunoassay

The 17-oxo-androgen EIA was used to calculate FAM concentrations following established methods (Palme and Mostl 1994). This had previously been used successfully to measure FAM concentrations in other mammal species (Mohle et al. 2002) including ungulates (Ganswindt et al. 2002, Hoby et al. 2006), and the cross-reactivity of the epiandrosterone antibodies has previously been reported (Palme and Mostl 1994). Serial dilutions of 24 pooled samples showed high parallelism with the standard curve ($p<0.001$), which had a LOD of 0.89 ng/g faeces. The intra-assay

CV was calculated at 4.55% across all samples, 4.85% amongst male samples and 4.26% amongst female samples. The overall inter-assay CV was calculated at 20.64%. Several assay plates were run per day, and given that previous studies had found assay date to account for significant variation between samples (e.g. Graham et al. 2010), the mean within-day inter-assay CV was also calculated to give a daily mean of 12.46% (± 1.80 SE).

2.7. FCM immunoassay

Cortisol is almost completely metabolised before excretion in the faeces (Ingram et al. 1999). Concentrations were therefore measured using a group-specific 11-oxoetiocholanolone EIA which had previously been validated in red deer using both ACTH (adrenocorticotropic hormone) challenge and natural disturbance tests (Huber et al. 2003b). The 11-oxoetiocholanolone EIA was used to calculate FCM concentrations following established methods (Palme and Mostl 1994), and the cross-reactivity of the 11-oxoetiocholanolone antibodies had previously been reported in Huber et al. (2003b). Serial dilutions of 24 pooled samples showed high parallelism with the standard curve ($p<0.001$), which had a calculated LOD of 3.51 ng/g faeces. The intra-assay CV was calculated at 4.16% across all samples, 4.01% amongst male samples and 4.31% amongst female samples. The overall inter-assay CV was calculated at 22.65%, and the mean within-day inter-assay CV was calculated at 15.02% (± 3.72 SE). The value of faecal over plasma hormone measures is discussed in chapter 1.4.

3 | Calf testosterone, maternal traits & survival

Calf testosterone- role of maternal traits and implications for survival[1]

[1] (Pavitt et al. 2014a)

3.1. Summary

This chapter investigates the potential role of maternal effects on offspring testosterone concentrations during the neonatal period, and the relationship between these testosterone levels and juvenile survival. Most of the variation among calves was accounted for by their age and sex. Both sexes showed a steep decline in testosterone levels within 24 hours of birth, although concentrations were consistently higher in males, and females showed a steeper decline in testosterone after 24 hours. Furthermore, male calves born in years after a brother had lower concentrations than those who were preceded by a sister or who were firstborns.

There was no evidence of repeatable differences among mothers in the testosterone levels of their calves, but there was significant among-year variation across the 17-year study period. Neonatal testosterone levels were also associated with calf survival, but only amongst individuals already at higher mortality risk: male calves born to first-time mothers were increasingly less likely to survive with higher neonatal testosterone concentrations. These results support the suggestion that a calf's circulating testosterone concentrations in early life could be linked to both individual and maternal characteristics, and that among-individual variation in these levels could have implications for juvenile fitness within a wild mammal population.

3.2. Introduction

Testosterone is known to act as an important biochemical mediator in the expression of many fitness and life history traits, and shows a high degree of variation between individuals within a population (Kempenaers et al. 2008, Williams 2008). It is positively associated with male reproductive traits (see reviews by Wingfield et al. 2001, Hau 2007 and references therein) and reproductive output (e.g. Reed et al. 2006), but negatively associated with female reproduction (e.g. Taitt and Krebs 1982, Staub and DeBeer 1997, Ketterson et al. 2005), as well as survival and immunocompetence in both sexes (Civantos 2002, Owen-Ashley et al. 2004, Cox et

al. 2005, Ketterson et al. 2005, Reed et al. 2006, Decristophoris et al. 2007, Malo et al. 2009, Mills et al. 2009).

Despite numerous fitness implications being shown, the causes of among-individual variation remain poorly understood, particularly in wild animal systems. In addition, the majority of studies have focussed on variation at the adult level, despite some evidence that early life testosterone levels can have both short- and long-term implications. Juvenile testosterone levels in lizards, for example, are negatively associated with growth and body condition during this life stage (Civantos 2002, Cox et al. 2005). Additionally, neonatal testosterone levels can have life-long implications for adult steroid synthesis (Gonzalez-Parra et al. 2000), and normal sexual development (Roffi et al. 1987, Matuszczyk et al. 1990, Foecking et al. 2005). Due to both the relative dearth of wild hormone studies and the limited research on early life steroid concentrations, this chapter first investigates the potential causes of variation in neonatal testosterone concentrations, and then considers the implications of this for juvenile survival.

3.2.1. Neonatal testosterone

Hormone concentrations in the first days of life are thought to be crucial to male sexual development (e.g. rats: Matuszczyk et al. 1990, see also Mann and Fraser 1996), and can change substantially across this short period. To date however, there has been little research on the causes of this variation or the implications for early life fitness, especially in mammals (though with some exceptions in birds: see Von Engelhardt and Groothuis 2011). Many reproductive hormones decline steeply within a neonatal mammal's first week (Lee et al. 1975, Sharma et al. 1984, Nakada et al. 2000), with some species exhibiting an intrinsic "testosterone surge" at or immediately after birth (e.g. rats, mice, horses and humans: Slob et al. 1980, Baum et al. 1988, see also Corbier et al. 1992 and references therein). This has only been found to a substantial degree in males where it is thought to serve a crucial role in their normal sexual development and de-feminisation (Roffi et al. 1987, Matuszczyk et al. 1990, Foecking et al. 2005). This supports evidence in the wider literature that neonatal testosterone levels play a key role in sexual differentiation and

development, with implications for the expression of adult traits in later life (Gonzalez-Parra et al. 2000, Seney et al. 2012).

Perinatal castration experiments show early high levels of testosterone to be of testicular origin in rats (Pfeiffer 1936), corroborating evidence from guinea pigs (Vreeburg et al. 1981, Despres et al. 1984) and cattle (Tapanainen 1983) which suggest that most foetal testosterone is endogenously produced by the foetus. This is not true for all species, however, as hyenas have been shown to convert maternal Δ^4-androstenedione (most likely of placental origin, Licht et al. 1992) into foetal testosterone during gestation (Licht et al. 1998). Finally, placental steroidogenesis has been recorded in a number of ruminants (Strauss et al. 1996, Hoffmann and Schuler 2002, Khatri 2011, Mondragon et al. 2012), making it feasible for placentally synthesised androgens to enter the foetal bloodstream. With testosterone of maternal origin, both sexes would be expected to be born with high concentrations that are rapidly depleted.

3.2.2. Maternal condition

Maternal condition during the prenatal and neonatal periods can have a significant impact on an individual's hormone profile and their subsequent development (see chapter 1.3.2), although effects in mammals are largely unknown. Previous studies of the wild Rum red deer show that male calves require a greater maternal investment than female calves: sons suckle more (Clutton-Brock et al. 1981) and are provided with a higher quantity and quality of milk (Landete-Castillejos et al. 2005). Mothers bearing sons also have a higher risk of winter mortality (Clutton-Brock et al. 1981), particularly amongst subordinate females (Gomendio et al. 1990), and are less likely to calve the subsequent year (Clutton-Brock et al. 1981) than those with daughters. Mothers are therefore likely to be in poorer condition after bearing a son, particularly if they do succeed in calving the following year. There is also evidence of humans being born lighter (Rickard et al. 2009 and references therein) and juvenile bighorn sheep (*Ovis canadensis*) being less likely to survive (Berube et al. 1996) when born after an older brother versus those following a sister or with no older siblings. This study tested for similar associations between condition-linked

traits in a mother (particularly her reproductive history and the sex of her previous calf) and the levels of testosterone in her new born calf.

3.2.3. Testosterone & survival

The possible consequences of among-individual testosterone variation were also considered because of the substantial and complex effects this can have on relative fitness. There can be both costs and benefits to high circulating testosterone concentrations which vary with age, sex and reproductive state. The benefits are primarily seen in reproductively active males, for example increased aggression and dominance (Lincoln 1972), antler morphology , and sperm quality (Malo et al. 2009) in red deer stags. Maintaining these high concentrations however, also has associated costs (Folstad and Karter 1992). High testosterone levels have been associated with immunosuppression, elevated parasite load, and reduced survival in adult males of various taxa (Owen-Ashley et al. 2004, Reed et al. 2006, Decristophoris et al. 2007, Malo et al. 2009), and with reduced growth rate and body condition in juvenile males (e.g. Civantos 2002, Cox et al. 2005). Females also suffer negative effects such as elevated glucocorticoids, reduced immune function and reduced survival (e.g. Taitt and Krebs 1982, Zysling et al. 2006, Gerlach and Ketterson 2013), as well as delayed/reduced reproduction (e.g. Clotfelter et al. 2004, Veiga and Polo 2008).

Using a subset of data from the Rum red deer population (calves born 1996-2012), this chapter investigates: (a) the degree to which individual calves differ from one another in their circulating testosterone concentrations, with particular reference to age and sex; (b) whether mothers in better condition produce calves with higher testosterone, particularly with regards to the sex of previous offspring; and (c) whether variation in testosterone level correlates with early life fitness measures, specifically survival to one year of age.

3.3. Methods

Circulating levels of testosterone were measured in 920 plasma sampled collected from red deer calves within 14 days of birth (see chapter 2.1.1 for details of sample

collection). Hormones were extracted from defrosted samples and assayed using a commercial EIA kit (see chapter 2.2 & 2.3 for full details of extraction and assay methodology). In 2013, 18 of these calves were bled twice to test whether capture affected testosterone levels: the first sample was taken immediately after capture and the section 4-5 minutes later when they are normally bled.

3.4. Statistical analyses

Unless otherwise noted, all analyses used mixed-effects models (package lme4: Bates et al. 2011) in R 2.14.2 (R.Development.Core.Team 2012). Maternal identity (n=333) and calf birth year (n=17) were included as random effects in all models to remove the impacts of pseudoreplication from shared maternity, and to account for annual variation in environmental conditions around birth. The sample assay date (n=7) was also fitted as a random effect in all testosterone response models as previous studies have found that assay date can account for significant variation among samples, possibly due to fluctuations in laboratory temperature (e.g. Graham et al. 2010, Watson et al. 2013). All analyses considered two separate models: the first comprised of just the main effects, whilst the second included two-way interactions. Models were run with both sexes together and then with each sex separately. The full models were fitted, and then non-significant variables sequentially dropped in a step-wise simplification based on the maximum likelihood estimates obtained from ANOVAs between nested models. The optimal model was accepted when all remaining effects were significant at $p<0.05$, and only significant interaction terms ($p<0.05$) are quoted. Testosterone was log-transformed to normalise the residuals, but is presented in the text as an unlogged mean (± 1 SE), and all continuous explanatory variables were centred prior to fitting the models.

3.4.1. Causes of neonatal testosterone variation

Variation in testosterone concentrations were explored using mixed effects models with normal errors. From the original 920 calves sampled, 62 calves were excluded due to missing data for one or more of the explanatory variables, and a further 4 removed for low repeatability of testosterone measures between duplicates (CV>10%). Analyses therefore included 854 individuals (from 333 mothers across 17

years). Models were fitted both to the overall dataset, and separately to female (n=434 calves from 237 mothers across 17 years) and male (n=420 calves from 236 mothers across 17 years) calves. These models considered the following variables as fixed effects: calf age, calf sex (except in sex-specific models), calf birth weight, maternal reproductive status (MRS), maternal age in years (linear and quadratic terms), the sex of the mother's previous calf, and the time of sample collection (see below for more detailed descriptions of all terms). Collection time was included to test for changes with time of day or the effect of time until freezing. Initially both linear and quadratic terms were fitted for collection time, but the quadratic effect was not significant (p=0.660) and so was excluded from the final models.

Calf-level fixed effects

Calf age at capture: The age of the calf when caught, estimated from the time the female last seen pregnant and the appearance and behaviour of the calf when captured. This was included as two separate variables:

(a) After 24 hours: A 2-level factor comparing calves (i) caught at/before or (ii) after 24 hours.

(b) Calf age (hours): A continuous variable measuring age (in hours) separately for calves caught before and after 24 hours. This nesting used the formula: after 24 hours + (after 24 hours * age (hours)) and significantly improved the fit of the model compared to one or other variable being fitted separately.

Calf sex: A 2-level factor for whether the focal calf was (i) female or (ii) male.

Calf birth weight: A continuous variable (measured in kg) estimated from calculations of weight increase per hour (the slope for capture weight regressed against capture age) based on the population mean. Mean weight increase per hour was multiplied by capture age (hours) and then subtracted from the weight of the individual at capture (Catchpole et al. 2004) using the formula: birth weight (kg) = capture weight - (0.016* capture age).

Testosterone: A continuous variable for circulating levels of testosterone. Testosterone was log-transformed using natural log, and (when used as an explanatory variable) adjusted for calf age and assay date by taking residuals from a regression against both variables.

Mother-level fixed effects

Maternal age: A continuous variable measured in years, denoting a female's age when she gave birth to the faecal calf. The quadratic term for age was fitted in addition to the linear term because a number of female reproductive traits are known to have a quadratic relationship with age in this population (see Nussey et al. 2009 and references therein).

MRS: A 3-level factor measuring the reproductive status of the mother at the time of the focal calf's birth. Females were classified as (i) naive mothers (not previously calved), (ii) milk hinds (produced a calf the previous year which survived to at least 1st October that year) or (iii) yelds (hind had calved before but not in the previous year, or did calve but offspring died <1st October). When considering the effects on survival, milk hinds and yelds did not vary from each other and so were collapsed into one factor level.

Sex of previous calf: A 2-level factor for the sex of the maternal sibling born prior to the focal calf (irrespective of the time gap between births). The previous sibling was either (i) "male" or (ii) "not-male". Calves with no previous sibling or who were born after a sister did not vary from each other and were collapsed into one factor level ("not-male").

3.4.2. Neonatal testosterone & calf survival

The date of death was estimated to within a week for most animals based on regular censuses (5 times per month for most months of the year), daily observations during the calving season (mid-May to mid-July), and mortality searches (Clutton-Brock et al. 1987, Catchpole et al. 2004). Calf survival to one year old was treated as a 2-level factor, with individuals either (i) surviving to one year of age or (ii) not. This was modelled using generalised linear mixed models (GLMM) with binomial error distributions. Of the original 920 calves sampled, 126 were omitted because they were either living but too young to be assigned a survival factor level (n=73 born in 2012), were shot before the age of one in managed culls at the edge of the study area (n=22), were missing data for one of more of the explanatory variables (n=27), or had poor repeatability of testosterone measures between duplicates (n=4). Separate models were fitted for (a) main effects only, and for (b) all second order interaction

terms involving testosterone. Survival was considered for both the overall dataset (n=794 calves from 304 mothers across 17 years), and for each sex individually (females: n=408 calves from 217 mothers across 17 years; males: n=386 calves from 216 mothers across 17 years). The fixed effects included in these models were: calf sex (except in sex-specific models), calf birth weight, maternal reproductive status (collapsed into naive versus non-naive mothers), maternal age in years (both linear and quadratic terms), and calf testosterone after log transformation and correction for calf age, collection time and the plasma sample assay date (see chapter 3.4.1 for more detailed descriptions of all terms).

3.5. Results

3.5.1. Effects of maternal & calf traits

Levels of testosterone in plasma collected immediately after capture did not differ from levels measured in the same individuals 4-5 minutes later (paired t-test: t=0.112, df=17, p=0.912). This suggests that variation in the time interval from start of catch to bleeding was insufficient to significant influence testosterone levels. Concentrations did, however, vary substantially among calves, with a mean of 386.70 pg/ml ± 11.85 SE and an overall range of 44.90-1858.43 pg/ml (n=854). Calf age had the biggest effect on testosterone concentrations (p<0.001; Table 3.1; Figure 3.1). Calves sampled during the first 24 hours of life had higher testosterone (509.63 pg/ml ± 17.43 SE, n=486) than those sampled afterwards (224.35 pg/ml ± 10.01 SE, n=368). Furthermore, whilst concentrations declined sharply from birth to 24 hours, they were less affected by calf age after 24 hours (Figure 3.1). Testosterone concentrations differed significantly between the sexes (p=0.002; Table 3.1a), with calf concentrations lower amongst females (358.36 pg/ml ± 15.17 SE, n=434) than males (423.52 pg/ml ± 18.50 SE, n=420).

Table 3.1 *Linear mixed models examining the main effects of calf and maternal traits on inter-individual variation in (log-transformed) testosterone amongst (a) all calves, (b) female calves, and (c) male calves. Significant effects are in bold.*

FIXED EFFECTS	**(a) All calves (854)**			**(b) Female calves (434)**			**(c) Male calves (420)**		
	Est. ± SE	*p*		Est. ± SE	*p*		Est. ± SE	*p*	
(intercept)	**6.366 ± 0.138**	***<0.001***	***	**6.373 ± 0.143**	***<0.001***	***	**6.512 ± 0.157**	***<0.001***	***
After 24 hrs	**-1.242 ± 0.073**	***<0.001***	***	**-1.091 ± 0.099**	***<0.001***	***	**-1.447 ± 0.111**	***<0.001***	***
Sex (male)	**0.114 ± 0.038**	***0.002***	**	-	-		-	-	
Birth weight	<-0.001 ± 0.016	*0.974*		0.001 ± 0.021	*0.946*		0.010 ± 0.024	*0.670*	
Maternal age	-0.001 ± 0.010	*0.198*		-0.001 ± 0.014	*0.228*		-0.001 ± 0.015	*0.947*	
Maternal age^2	0.001 ± 0.002	*0.501*		0.003 ± 0.003	*0.173*		<0.001 ± 0.003	*0.981*	
MRS (*naive*) [a]	-0.063 ± 0.079	*0.767*		-0.104 ± 0.107	*0.316*		-0.031 ± 0.118	*0.171*	
MRS (*yeld*) [a]	0.013 ± 0.044			-0.092 ± 0.060			0.096 ± 0.065		
Sex of previous calf (♂)	**-0.117 ± 0.042**	***0.012***	*	-0.066 ± 0.057	*0.248*		**-0.164 ± 0.062**	***0.021***	*
Collection time	**-0.025 ± 0.007**	***<0.001***	***	-0.012 ± 0.009	*0.199*		**-0.036 ± 0.010**	***<0.001***	***
Calf age (hrs) <24 hrs	**-0.046 ± 0.003**	***<0.001***	***	**-0.042 ± 0.004**	***<0.001***	***	**-0.052 ± 0.005**	***<0.001***	***
Calf age (hrs) >24 hrs	**-0.001 ± 0.001**			**-0.002 ± 0.001**			**<0.001 ± 0.001**		
RANDOM EFFECTS	**Var.**	***p***		**Var.**	***p***		**Var.**	***p***	
Mother ID (n=333/237/236)	0.012	*0.191*		0.014	*0.359*		0	*1.000*	
Birth year (n=17)	**0.015**	***<0.001***	***	**0.011**	***0.017***	*	**0.024**	***0.007***	**
Date of assay (n=7)	**0.097**	***<0.001***	***	**0.089**	***<0.001***	***	**0.106**	***<0.001***	***
Residual	0.275			0.240			0.310		

(p<0.1="."; p<0.05=""; p<0.01="**"; p<0.001="***")*

[a] *MRS: values are relative to milk hinds*

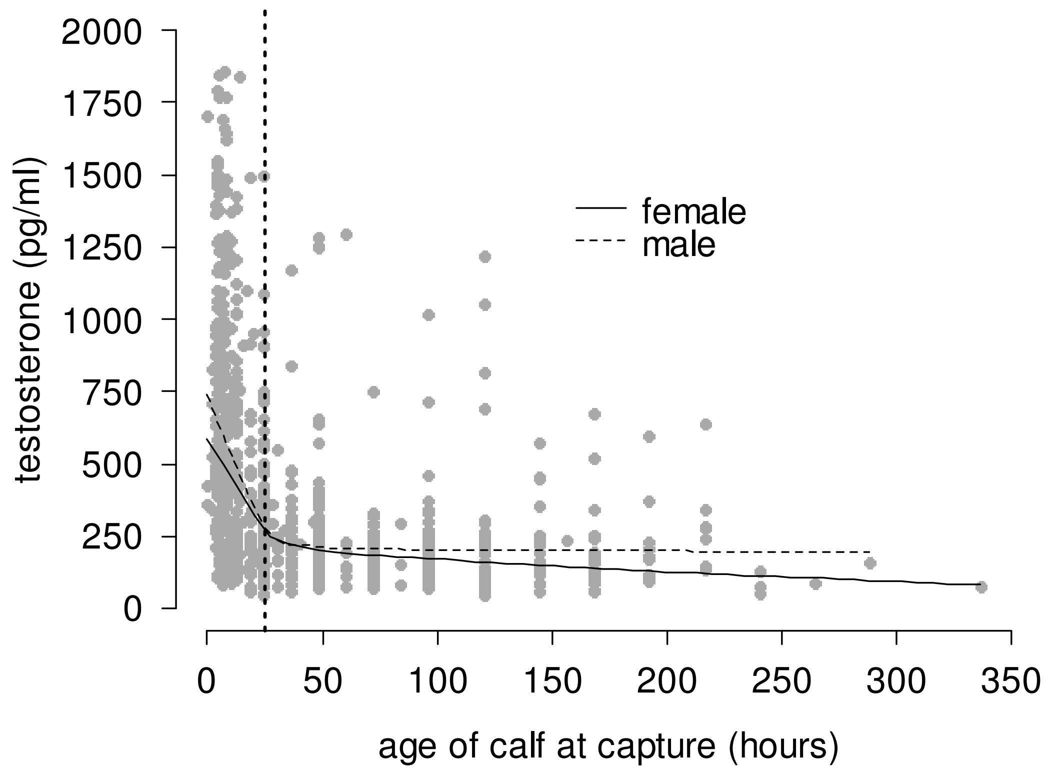


Figure 3.1: *Variation in testosterone concentration with calf age. The points are the raw data, and smoothing lines for female and male calves were fitted from the models in Table 3.1 to illustrate the variation in testosterone with age. 24 hours is indicated by a vertical dashed line.*

One significant interaction was identified between calf age and sex (p=0.011; Table 3.2; Figure 3.1). Levels of testosterone declined faster in females sampled after 24 hours old than in males of the same age group, although concentrations did not differ between the sexes amongst the calves sampled within 24 hours. This interaction appeared to be driven by 13 older calves (females: n=4; males: n=9) sampled >200 hours old, and when these were removed the interaction was no longer significant (p=0.140).

***Table 3.2**: Linear mixed model examining the interaction effects of calf and maternal traits on inter-individual variation in (log-transformed) testosterone amongst all calves (n=854). Significant terms are in bold, and all estimates are quoted from models containing all main and significant interaction terms.*

FIXED EFFECTS	All calves (845)		
	Est. ± SE	*p*	
(intercept)	6.307 ± 0.144		
After 24 hours	-1.055 ± 0.103		
Calf age (hours) < 24 hours	-0.041 ± 0.004		
Calf age (hours) >24 hours	-0.002 ± 0.001		
Sex (♂)	0.230 ± 0.095		
Birth weight	<0.001 ± 0.016		
Maternal age	-0.001 ± 0.010		
Maternal age^2	0.001 ± 0.002		
MRS (*naive*) [a]	-0.057 ± 0.079		
MRS (*yeld*) [a]	-0.012 ± 0.044		
Sex of previous calf (♂)	-0.115 ± 0.042		
Collection time	-0.026 ± 0.007		
After 24 hrs : Sex (♂)	**-0.327 ± 0.145**	***0.010***	*
Calf age (hrs) <24 hrs : Sex (♂)	**-0.009 ± 0.006**	***0.011***	*
Calf age (hrs) >24 hrs : Sex (♂)	**0.003 ± 0.001**		
RANDOM EFFECTS	**Var.**	***p***	
Mother ID (n=333)	0.012	*0.251*	
Birth year (n=17)	**0.016**	***<0.001***	***
Date of assay (n=7)	**0.096**	***<0.001***	***
Residual	0.242		

(p<0.1="."; p<0.05=""; p<0.01="**"; p<0.001="***")*

[a] *MRS: values are relative to milk hinds*

The sex of the previous calf had a significant effect on an individual's circulating testosterone (p=0.012; Table 3.1a). Concentrations were lower amongst calves that were born after an older brother compared to those that were not (361.53 pg/ml ± 18.16 SE, n=321 versus 407.92 pg/ml ± 15.73 SE, n=533). Whilst similar in both sexes (test for interaction between calf sex and the sex of their previous sibling: p=0.236), this relationship only remained significant amongst male calves (p=0.021; Table 3.1c) when the sexes were considered separately.

Finally, calf testosterone also declined with collection time, with testosterone levels being lower from samples collected later in the day ($p<0.001$). This did not differ significantly between the sexes (test for interaction between calf sex and collection time: $p=0.087$), but only remained significant amongst male calves when the sexes were examined separately (females: $p=0.199$; males: $p<0.001$). No other main effects were significant. Of the random effects, assay date had the largest effect ($p<0.001$), however testosterone also varied significantly among birth cohorts in all models ($p<0.05$; Table 3.1), and mean annual testosterone concentration was strongly correlated between the two sexes ($r^2=0.59$, $n=17$). Whilst there was no evidence of significant variation among mothers, maternal identity was retained in the models to avoid pseudoreplication of maternal variables.

3.5.2. Neonatal testosterone & calf survival

Adjusted for calf age and assay date, circulating testosterone levels had no main effect on the probability of a calf surviving to the age of one (Table 3.3). The most significant effect in all survival models was the estimated weight of the calf at birth ($p<0.001$; Table 3.3), with heavier calves having a higher probability of survival. Older mothers were also less likely to have a calf surviving to one year old ($p=0.014$; Table 3.3a); however, when the sexes were examined separately this relationship was only significant in male calves (females: $p=0.179$; males: $p<0.001$; Table 3.3). Males were also less likely to survive when born to naive mothers ($p=0.003$; Table 3.3c), whilst maternal naivety had no effect on female calf survival ($p=0.204$; Table 3.3b). The removal of birth weight from the models did not alter the overall significant variables, showing their effects to be independent of birth weight.

Table 3.3: *Generalised linear mixed models examining the main effects of calf and maternal traits on inter-individual variation in calf survival amongst (a) all calves, (b) female calves, and (c) male calves. Significant effects are in bold.*

FIXED EFFECTS	(a) All calves (794)			(b) Female calves (408)			(c) Male calves (386)		
	Est. ± SE	*p*		Est. ± SE	*p*		Est. ± SE	*p*	
(intercept)	**1.087 ± 0.313**	***0.001***	***	**0.746 ± 0.236**	***<0.001***	***	**1.020 ± 0.333**	***0.001***	***
Sex (male)	-0.251 ± 0.191	*0.201*		-	-		-	-	
Birth weight	**0.601 ± 0.092**	***<0.001***	***	**0.537 ± 0.107**	***<0.001***	***	**0.654 ± 0.123**	***<0.001***	***
MRS (*naive*) [a]	-0.168 ± 0.364	*0.640*		0.583 ± 0.465	*0.204*		**-1.192 ± 0.533**	***0.003***	**
Maternal age	**-0.067 ± 0.050**	***0.014***	*	0.025 ± 0.061	*0.179*		**-0.180 ± 0.071**	***<0.001***	***
Maternal age^2	-0.010 ± 0.010	*0.093*	.	-0.014 ± 0.011	*0.519*		-0.001 ± 0.015	*0.948*	
Testosterone	-0.034 ± 0.173	*0.846*		-0.076 ± 0.229	*0.740*		0.027 ± 0.230	*0.581*	
RANDOM EFFECTS	**Var.**	***p***		**Var.**	***p***		**Var.**	***p***	
Mother ID (n=304/217/216)	**1.274**	***<0.001***	***	0.432	*0.130*		0.593	*0.139*	
Birth year (n=17)	**1.083**	***<0.001***	***	**0.431**	***0.003***	**	**1.170**	***<0.001***	***

(p<0.1="."; p<0.05=""; p<0.01="**"; p<0.001="***")*

[a] *MRS: values are relative to experienced hinds (hinds that have calved before)*

When considering two-way interactions between testosterone and other main effects on calf survival, there was a relationship between testosterone, maternal experience and survival (see Figure 3.2a & b). Amongst the offspring of naive mothers, higher neonatal testosterone was associated with lower calf survival, but there was no relationship if the mother had calved before (p=0.016; Table 3.4a; Figure 3.2a & b). Whilst this trend was present in both sexes when considered individually, it was only significant amongst male calves (females: p=0.287; males: p=0.045; Table 3.4b & c, Figure 3.2c-f).

Table 3.4: *Generalised linear mixed models examining the effects of calf and maternal traits and any interaction effects with testosterone concentrations on inter-individual variation in calf survival amongst (a) all calves, (b) female calves and (c) male calves. Estimates are quoted from maximal models containing all main effects and two-way interactions with testosterone. Significant effects are in bold.*

FIXED EFFECTS	(a) All calves (794)			(b) Female calves (408)			(c) Male calves (386)		
	Est. ± SE	*p*		Est. ± SE	*p*		Est. ± SE	*p*	
(intercept)	1.089 ± 0.318			0.760 ± 0.237			1.017 ± 0.346		
Sex (male)	-0.244 ± 0.193			-			-		
Birth weight	0.620 ± 0.093			0.542 ± 0.237			0.714 ± 0.128		
MRS (*naive*) [a]	-0.183 ± 0.370			0.608 ± 0.469			-1.309 ± 0.554		
Maternal age	-0.067 ± 0.050			0.027 ± 0.061			-0.193 ± 0.073		
Maternal age^2	-0.010 ± 0.010			-0.015 ± 0.011			0.003 ± 0.016		
Logged testosterone (residuals)	-0.023 ± 0.316			-0.008 ± 0.313			0.149 ± 0.317		
Testosterone: Sex (male)	0.225 ± 0.357	*0.528*		-	-		-	-	
Testosterone: Birth weight	-0.099 ± 0.153	*0.547*		-0.095 ± 0.198	*0.622*		-0.157 ± 0.208	*0.452*	
Testosterone: MRS (*naive*) [a]	**-1.650 ± 0.683**	***0.016***	*	-0.575 ± 0.827	*0.287*		**-2.584 ± 1.026**	***0.045***	*
Testosterone: Maternal age	-0.097 ± 0.086	*0.549*		0.012 ± 0.113	*0.765*		0.171 ± 0.116	*0.341*	
Testosterone: Maternal age^2	0.018 ± 0.018	*0.265*		0.004 ± 0.022	*0.857*		0.027 ± 0.025	*0.208*	
RANDOM EFFECTS	**Var.**	***p***		**Var.**	***p***		**Var.**	***p***	
Mother ID (n=304/217/216)	**1.353**	***<0.001***	***	0.449	*0.138*		0.645	*0.126*	
Birth year (n=17)	**1.112**	***<0.001***	***	**0.434**	***0.003***	**	**1.277**	***<0.001***	***

(p<0.1="."; p<0.05=""; p<0.01="**"; p<0.001="***")*

[a] *MRS: values are relative to experienced hinds (hinds that have calved before)*

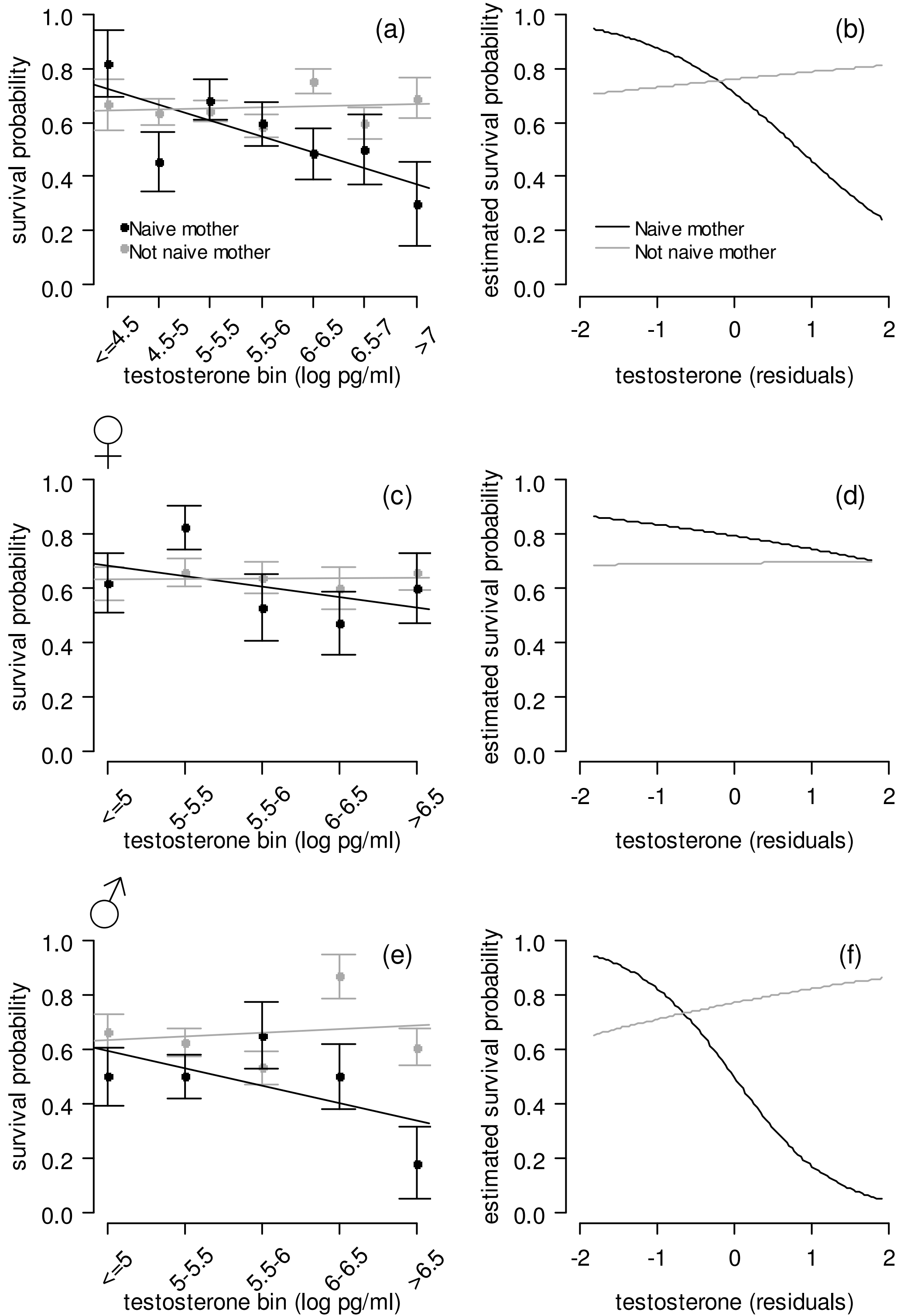


***Figure 3.2**: The probability of overall (a & b), female only (c & d) and male only (e & f) calf survival with varying testosterone concentrations for the offspring of naive versus experienced mothers, showing the mean survival probability ± SE for bins of log-transformed testosterone (a, c & e), and the survival predictions estimated from the maximal model shown in Table 3.4 (b, d & f).*

3.6. Discussion

This study presents the first estimates of variation in testosterone levels in new born mammals in a wild population. Testosterone differed according to several, predominantly calf, traits, but any implications of this variation for subsequent survival were only apparent in a subset of the data.

3.6.1. Age effects

High testosterone levels amongst the youngest animals (Figure 3.1) could either result from mother-to-offspring transfer at some point prior to birth, or from endogenous production by the foetus or new born calf. Whilst the transfer of testosterone from mother to offspring is well documented in birds (Von Engelhardt and Groothuis 2011), results are mixed in mammals. Experimental studies in both rats (Slob et al. 1983, Sathishkumar et al. 2011, Sun et al. 2012) and guinea pigs (Vreeburg et al. 1981) suggest that the placenta acts as a barrier to testosterone transfer, however hyenas have been shown to convert maternal androgens into foetal testosterone during gestation (Licht et al. 1998).

This study was not sufficient to conclusively identify the source of the high neonatal testosterone concentrations observed. A testosterone surge immediately after birth would have provided strong evidence for endogenous testosterone production in early life, however this time period was not sufficiently covered by the dataset. These surges tend to peak within 2 hours of neonatal life (Corbier et al. 1992), but few calves were sampled younger than 3 hours, thus missing the critical time period for detection. Testosterone surges do, however, tend to occur in males and not females (see Corbier et al. 1992). Thus, the lack of sex difference in the rate of decline of testosterone with age during the first 24 hours (Figure 3.1) suggests a testosterone surge was not the cause of high testosterone levels among the youngest animals in this study. Instead, both sexes may have been born with relatively high testosterone, either as a result of endogenous *in utero* production (which has been observed in early- to mid-term male but not female foetuses Lincoln 1973), or transfer from the mother. Whilst maternally derived testosterone is possible (e.g. Licht et al. 1998),

studies in both rodents and cattle suggest that it is more likely to be intrinsically produced by the calf (e.g. Slob et al. 1980, Vreeburg et al. 1981, Tapanainen 1983). Concentrations in calves with shared maternities were also no more similar than other calves, despite evidence from other species suggesting that an individual's steroid concentrations may be repeatable between years (Williams 2008, While et al. 2010). Furthermore, there were few substantial effects of variable maternal traits (e.g. maternal age) which could have explained why the effect of the mother differed between years, although it is possible that cohort variation (possibly the result of varying weather conditions) masked any effect of shared maternity. At present however, and without access to samples from the time of birth, the source of the high neonatal testosterone measures remains unresolved.

3.6.2. Effects of maternal condition

Maternal traits appear to have only a limited association with offspring testosterone; the only significant indication of an effect of maternal condition was the sex of the previous calf. Little is known about the effects of older sibling traits on an individual's endocrinology, but this study found that male calves born after a brother had lower levels of circulating testosterone than other male calves. Although less statistically significant than calf sex ($p=0.012$ versus $p=0.002$; Table 3.1a), the estimated effect size of previous sibling sex was similar in magnitude to that of sex (Table 3.1). These results are also in accordance with other findings of sequential sibling effects on testosterone in humans: boys (but not girls) born within four years of their preceding sibling had lower neonatal testosterone concentrations than those born after four years or to naive mothers; however, this study did not consider the sex of that previous sibling (Maccoby et al. 1979). The older brother effect also agrees with previous work on this red deer population which show sons to be more costly to their mothers than daughters (Clutton-Brock et al. 1981, Gomendio et al. 1990, Landete-Castillejos et al. 2005). Early/mid-term male (but not female) foetuses (<180 days old) have also been shown to produce testosterone *in utero* (Lincoln 1973) which they may transfer to the mother during development (Meulenberg and Hofman 1991), with potential negative effects on her condition as a result (Clotfelter et al. 2004, Ketterson et al. 2005, Gerlach and Ketterson 2013).

If calf testosterone is intrinsically produced (e.g. Pfeiffer 1936, Vreeburg et al. 1981, Tapanainen 1983, Despres et al. 1984, though see Licht et al. 1998) then the implication is that the observed sibling effect is actually reflecting the effect of a mother's condition on her calf's production of testosterone. Given that maternal condition is known to influence other effects such as birth weight in the deer (Coulson et al. 2003), it is perhaps surprising that there was no correlation between testosterone concentration and birth weight. The different relationships between maternal condition and resulting calf traits may, however, be underpinned by different mechanisms: further research is clearly needed in this area before any mechanistic conclusions can be drawn.

Further to this sibling effect, there was also significant among-cohort variation in calf testosterone levels, especially in males, which may reflect maternal condition prior to birth. Whilst testosterone is known to undergo photoperiodic cycles in red deer (e.g. Lincoln and Kay 1979, Suttie et al. 1989), there is little research on the effect of annual variation in environmental conditions at a given time of year. Weather could both directly and indirectly affect maternal condition, with potential implications for a female's ability to invest in a developing foetus, however further research is needed.

3.6.3. Collection time

The negative relationship between testosterone and collection time could either be biological (i.e. diurnal rhythms, as observed in red deer by Lincoln and Kay 1979) or methodological in origin. On average, testosterone levels in adult male fallow deer (*Dama dama*) are shown to be highest during the early afternoon, with concentrations declining into the evening (Asher and Peterson 1991). Increased testosterone concentrations during the early afternoon are consistent with the patterns seen in this study where most samples were collected after midday. Given that samples were not refrigerated until the evening of collection, the alternative explanation is that a decrease in testosterone with time of day represents an increase in testosterone concentration with the amount of time a sample was kept at ambient

temperature. This trend is consistent with studies of human blood which show testosterone concentrations to be higher in samples that have spent longer at ambient temperature (Jones et al. 2007). This is attributed to the enzymatic conversion of other androgens (e.g. androstenedione) to testosterone, or the increased decoupling of testosterone from sex hormone binding globins (see Jones et al. 2007 for discussion).

3.6.4. Neonatal testosterone & calf survival

No evidence of any overall association was found between calf survival and neonatal testosterone levels amongst all calves. Survival did, however, decline with increased testosterone amongst the sons of naive mothers, whilst survival amongst those born to experienced mothers showed no trend. This study shows that firstborn males were already more likely to die in their first year than those whose mother had calved before. Naive mothers have no prior experience of raising a calf and, being young, are likely also have fewer resources to invest in their offspring (Landete-Castillejos et al. 2004). Given that maternal investment is higher for a son versus a daughter (Clutton-Brock et al. 1981, Landete-Castillejos et al. 2005), firstborn male calves are particularly vulnerable to mortality. Considering that firstborn males already had a higher rate of mortality than non-firstborn males, and are therefore also likely to be in poorer condition, the additional costs of high testosterone (e.g. Folstad and Karter 1992) may be sufficient to reduce survival further. High testosterone has previously been linked to reduced growth and body condition in juvenile lizards (Civantos 2002, Cox et al. 2005) as well as elevated parasite load, increased basal metabolic rate, immunosuppression and reduced survival in adult males across multiple taxa (e.g. lizards: Marler and Moore 1988, birds: Buchanan et al. 2001, Owen-Ashley et al. 2004, mammals: Decristophoris et al. 2007, Malo et al. 2009). There is also evidence from other species that early life testosterone levels may have important implications for the expression of adult traits later in life (Pfaff and Zigmond 1971, Christensen and Gorski 1978, Seney et al. 2012), including the secretion of steroid hormones (Gonzalez-Parra et al. 2000), however there was not scope to test this here.

As with previous studies on the Rum red deer study population, heavier calves in these analyses were more likely to survive than lighter ones (e.g. Walling et al. 2011). Maternal age has also been shown to be an important determinant of calf survival, which was highest in the offspring of middle aged mothers (e.g. Nussey et al. 2009). Whilst this study found the same pattern between survival and the quadratic of maternal age, the quadratic term was not statistically significant in any of the models (Table 3.3); instead survival declined linearly with maternal age. The repeatability of calf survival between mothers (Table 3.3) has also previously been documented for this study population (Nussey et al. 2009, Walling et al. 2011). Conversely, there was no significant sex difference in survival, an effect which had been significant in many previous studies (e.g. Catchpole et al. 2004). Such inconsistencies could be due to this study only using a subset (years: 1996-2012) of the data available for the Rum red deer rather than the full dataset which extends from the early 1970s. Other studies of this population have also found differences between subset versus full dataset (e.g. Walling et al. 2011, although this was likely due to birth weight effects).

3.6.5. Conclusion

In summary, testosterone concentrations varied substantially among neonatal red deer calves. The highest concentrations were found in the youngest animals, although contrary to findings from other mammal species there was no evidence of a sex-specific testosterone surge after birth in this red deer population. These results highlight the importance of considering the potential effects of previous siblings, particularly when their characteristics can influence the condition of the mother. Whilst testosterone was only linked to survival in individuals already at a greater risk of mortality (firstborn males), these results do indicate that testosterone, known to affect adult fitness, may also have an effect on juvenile fitness.

4 | The heritability of neonatal testosterone concentrations[2]

[2] (Pavitt et al. 2014b)

4.1. Summary

Testosterone is an important hormone which has been shown to have sex-specific links to fitness in numerous species. Although testosterone concentrations vary substantially between individuals in a population, little is known about its heritable genetic basis or between-sex genetic correlations, which determine its evolutionary potential. This chapter found circulating calf testosterone levels to be both heritable (0.16 ± 0.06 SE) and correlated between the sexes (0.94 ± 0.65 SE) in wild red deer calves. This may have important evolutionary implications if, as in adults, the sexes have divergent optima for circulating testosterone levels.

4.2. Introduction

After exploring potential individual and maternal effects on a calf's circulating testosterone levels (chapter 3), the cause of considerable variation remained unresolved. The analyses in chapter 4 built on these findings from chapter 3 to explore the potential for a heritable genetic component to explain some of this remaining variation between individual testosterone levels.

Understanding the degree to which an individual's hormone concentrations are determined by their underlying genetics is important in ascertaining the potential for these selection pressures to change mean population levels. There is certainly ample scope for directional selection to act on this trait in adult males, given that high concentrations of testosterone are linked to enhanced reproductive traits (Zuk et al. 1995, Lank et al. 1999, Malo et al. 2009) as well as an overall net increase in male reproductive output (e.g. though extra-pair copulation: Reed et al. 2006). The associated costs to both sexes (Civantos 2002, Owen-Ashley et al. 2004, Cox et al. 2005, Ketterson et al. 2005, Reed et al. 2006, Decristophoris et al. 2007, Malo et al. 2009, Mills et al. 2009) may, however, prevent optimal concentrations for male reproduction being met. Although most studies of inter-individual testosterone variation have focussed on adults, evidence of a negative effect of high testosterone concentrations on male calf survival in chapter 3 (Figure 3.2) suggests that there is also scope for neonatal levels to be under selection.

4.2.1. Heritability of neonatal testosterone concentration

Evidence from a range of species suggests that testosterone levels are indeed heritable (Table 4.1), although this has rarely been investigated in the wild (see Williams 2008). To date, most studies have predominantly focussed on humans (mean h^2: 0.45; range: 0-0.91; Table 4.1) or captive/laboratory populations (though see King et al. 2004, Tschirren et al. 2009) which often use artificially-selected lines (e.g. Robinson et al. 1994, Schroderus et al. 2010, Okuliarova et al. 2011). Studying heritability in captive animals often allows for a higher level of environmental control, but may not be representative of free-roaming and unmanaged systems. In the wild, populations are exposed to unpredictable and changing environmental conditions (see Calisi and Bentley 2009 for further discussion), which may also influence hormone concentrations and reduce heritability estimates as a result (Calisi and Bentley 2009).

There are also other limitations to our current understanding of testosterone heritability. Most studies to date have focussed on adult concentrations (though see Table 4.1 for exceptions in humans and garter snakes), however hormone concentrations can also vary substantially amongst young animals, with potential fitness consequences as a result (chapter 3). Previous analyses of hormone heritability have also focussed on the similarity between pairs of relatives (e.g. parent-offspring regressions or sibling relationships; see Table 4.1), which provide an estimate based on limited data and risk inflating heritability estimates due to shared environmental or maternal effects (Kruuk and Hadfield 2007). Methods have, however, been developed which avoid many of the problems associated with paired-relative regressions. The animal model is a particular type of mixed-effect model which allows the use of complex multi-generational pedigrees to estimate heritability. These models do not just rely on a single type of relationship (e.g. between parent and offspring), but use measures of relatedness between all individuals in the pedigree to estimate similarities between relatives. This is a powerful tool which is robust to incomplete data such as unknown sires, a problem prevalent in wild animal studies where there is no paternal care and paternity is not

immediately apparent (see Kruuk 2004, Wilson et al. 2010 for discussion of the animal model in ecology). The use of relatedness information across complex pedigrees both reduces the potential for bias, and allows more efficient use of the data typical of natural populations (Kruuk 2004, Kruuk and Hadfield 2007).

Table 4.1: *Estimated h^2 of testosterone concentrations across a range of species (± estimates of error where given).*

Species	Sample	Method	Sex	Wild/captive	h^2	Reference
BIRDS						
Collared flycatcher *(Ficedula albicollis)*	Yolk	Mother-daughter	F	Wild	0.75 (0.23-1.26CI)	Tschirren *et al*. (2009)
Japanese quail *(Coturnix japonica)*	Yolk	Mother-daughter	F	Captive selection lines	0.42 ± 0.03 SD	Okuliarova *et al*. (2011)
MAMMALS						
Bank vole	Blood	Sire-midson	M	Lab (wild-born)	0.58 ± 0.16 SE	Mills *et al*. (2009)
(*Myodes glareolus*)	Blood	Pedigree	M	Captive selection lines	0.24	Schroderus *et al*. (2010)
Domestic pig	Blood	Sire-son	M	Domestic	0.37 ± 0.16 SD	Lubritz *et al*. (1991)
(*Sus domesticus*)	Blood	Paternal half-sib	M	Domestic	0.14 ± 0.44 SE	Bates *et al*. (1986)
	Blood	Sire-son	M	Captive selection lines	0.37 ± 0.16 SE	Robinson *et al*. (1994)
Human						
Newborn	Blood	MZ & DZ same-sex twins	M & F	-	0	Sakai *et al*. (1992)
Baby	Saliva	Same- & mixed-sex twins	M & F	-	0	Caramaschi *et al*. (2012)
Adolescents	Blood	MZ twins	M	-	0.66 (0.44-0.79CI)	Harris *et al*. (1998)
	Blood	MZ twins	F	-	0.60 (0.21-0.78CI)	Harris *et al*. (1998)
	Blood	DZ same-sex twins	M	-	0.34 (0.02-0.57CI)	Harris *et al*. (1998)
	Blood	DZ same-sex twins	F	-	0.01 (-0.39-0.34CI)	Harris *et al*. (1998)
	Saliva	Same- & mixed-sex twins	M & F	-	0.52	Hoekstra *et al*. (2006)
Adults	Blood	MZ & DZ twins	M	-	0.26	Meikle *et al*. (1986)
	Blood	MZ & DZ twins	M	-	0.56 (0.36-0.71CI) – 0.65 (0.48-0.77CI)	Sluyter *et al*. (2000)
	Blood	MZ & DZ twins (59-70 y.o.)	M	-	0.57 (0.46-0.68 CI)	Ring *et al*. (2005)
	Blood	Twin & sibling pairs	M	-	0.58 (0.27-0.76 CI)	Kuijper *et al*. (2007)
	Blood	Twin & sibling pairs	F	-	0.39 ± 0.14 SE	Stone *et al*. (2009)

	Blood	Sibling pairs	M	-	Total t: 0.65 ± 0.10 SE Free t: 0.54 ± 0.10 SE	Bogaert *et al.* (2008)
	Blood	Sibling pairs	F	-	0.46	Franks *et al.* (2008)
	Blood	Sire-son	M	-	0.40 ± 0.50 SE	Travison *et al.* (2014)
	Blood	Parent-offspring & sibling pairs	M & F	- -	Male: 0.91 Female: 0.65	Hong *et al.* (2001)
	Blood	Mother-daughter & sibling pairs	F	-	0.26 ± 0.05 SE	Coviello *et al.* (2011)
REPTILES						
Garter snake (*Thamnophis sirtalis*)	Blood	Male full sib	M	Wild-caught mothers		King *et al.* (2004)
		195 days old			0.2 (-0.24-0.76 CI)	
		318 days old			1.18 (0.72-1.56CI)	
		437 days old			0.91 (0.38-1.39CI)	

M: Male; F: Female

MZ: monozygotic; DZ: dizygotic

4.2.2. Cross-sex genetic correlation

In addition to knowing relatively little about the heritability of testosterone in wild species, even less is known about the degree of genetic correlation between male and female testosterone concentrations. To date, this has not been directly estimated outside of humans, where a positive cross-sex genetic correlation was recorded in a study of 12 year-old twins (Hoekstra et al. 2006). Although not directly estimated, the presence of a positive cross-sex genetic correlation has also been inferred in adult mammals (Schroderus et al. 2010, Mills et al. 2012), birds (Ketterson et al. 2005, Moller et al. 2005), and fish (Mank 2007). A genetic correlation indicates a shared genetic basis for the traits in question (in this case male and female testosterone concentrations) which would prevent them from evolving independently. This is important for adults because whilst both sexes have been shown to suffer survival and immunity costs as a consequence of high levels of testosterone (Taitt and Krebs 1982, Owen-Ashley et al. 2004, Reed et al. 2006, Zysling et al. 2006, Decristophoris et al. 2007, Malo et al. 2009, Gerlach and Ketterson 2013), the relationship between testosterone concentration and reproductive fitness differs between them. In males, high levels of testosterone have been linked to enhanced reproductive traits (e.g. in deer stags: Lincoln et al. 1972, Malo et al. 2009), and increased overall reproductive output in birds (Reed et al. 2006)(Reed et al. 2006). In females, however, the opposite appears true, with high testosterone levels associated with delayed or reduced reproduction (e.g. Clotfelter et al. 2004, Veiga and Polo 2008). Males are therefore expected to be under selection for higher concentrations (e.g. Ketterson et al. 2005, Moller et al. 2005, Schroderus et al. 2010), whilst lower concentrations are expected to be favoured in females. Where these sexually-antagonistic selection pressures exist, a shared genetic architecture (i.e. a positive genetic correlation, Lande 1987) would constrain either sex from reaching their optimum. This is further discussed in chapter 1.2.2.

Testosterone levels may also be important to sex-specific trait development in early life (Lummaa et al. 2007 and references therein). In rats, for example, males with suppressed testosterone during the neonatal stage developed more feminised

behaviour in adult life (Pfaff and Zigmond 1971, Corbier et al. 1983, Roffi et al. 1987, Motelica-Heino et al. 1993), whilst females exposed to high levels of testosterone as neonates exhibited masculinised behaviour as adults (Pfaff and Zigmond 1971, McDonald and Doughty 1972, Christensen and Gorski 1978). This again suggests sexually-antagonistic selection pressures which could constrain trait evolution in the presence of a positive genetic correlation, although to our knowledge early life sexually antagonistic selection remains little explored at the inter-individual level.

In chapter 3, neonatal testosterone was shown it to be dependent on the age and sex of the calf, as well as the time of day it was sampled and the sex of its preceding maternal sibling. This chapter extends these analyses to investigate: (a) the heritability of calf testosterone concentrations; and (b) the genetic correlation in calf testosterone concentrations between the sexes.

4.3. Methods

In this chapter, the data in chapter 3 (n=854 calf testosterone concentrations) were further analysed to explore the potential for an individual's genes to account for additional variation in circulating testosterone levels. These studies utilise the same set of circulating testosterone measures as those used in chapter 3, and account for the factors previously identified as explaining significant inter-individual variation in these hormone measures.

4.3.1. Pedigree

Within the Rum red deer study population, maternal and paternal identification can be made based on a combination of behavioural and genetic data (Walling et al. 2010). The pedigree based on raw data used in these analyses included 4482 individuals where at least one direct relative was known. Calves remain with their mother for several months until they are weaned, allowing maternity to be assigned to 3741 calves (from 801 mothers) based on observations of mother-calf behaviour (e.g. suckling) or of the birth itself. Paternity was assigned to 2190 calves (from 396 sires) using both MasterBayes (Hadfield et al. 2006) and COLONY2 (Wang 2004, Wang and Santure 2009) programmes. Candidate sires were considered based on a

combination of male rutting behaviour and genetic analysis which utilised up to 15 microsatellite loci (see Walling et al. 2010 for more detailed description of parentage assignment). Of the 854 calves with known neonatal testosterone concentrations used in this study, maternity was known for all calves and paternity had been assigned to 786 calves. In total, the informative pedigree for these calves contained 1293 maternal links and 1137 paternal links, had a modal depth of 5 generations, and a maximum depth of 9 generations.

4.4. Statistical analyses

4.4.1. Heritability of neonatal testosterone concentration

Calf testosterone concentration (V_P) was log-transformed (to normalise residuals) and analysed using an univariate animal model in ASReml-R 3.0.3 (package: asreml, Butler 2009). The following fixed effects were included in the model because chapter 3 showed them to explain significant amounts of among-individual variation in calf testosterone levels: calf age at capture, calf sex, sex of mother's previous calf, collection time, and assay date (see chapter 3.4.1 for full description).

Failure to include fixed effects which are known to be important would elevate the unknown residual variance and therefore reduce the proportion of overall phenotypic variance accounted for by additive genetic variance. After accounting for these fixed effects, variance in testosterone concentration (V_P) was partitioned into three components: additive genetic variance (V_A), birth year (V_{YOB}), and the remaining unexplained residual variance (V_R). Variance due to maternal effects could be distinguished from V_A for other traits in this population (Kruuk and Hadfield 2007), however in this study maternal identity was tested but excluded because effects were negligible (p=1). The final model therefore partitioned V_P into $V_A + V_{YOB} + V_R$.

Random effects were tested for significance using likelihood ratio tests (LRTs) of models with and without each effect, assuming a chi-squared distribution with 1 degree of freedom. h^2 was calculated as the proportion of V_P which was accounted for by V_A (Falconer and Mackay 1996).

4.4.2. Cross-sex genetic correlation

A bivariate model was then fitted to estimate components of (co)variance between male (n=420) and female (n=434) testosterone concentrations. Because there was greater phenotypic variance in male than female levels (chapter 3; Table 3.1), the sex-specific (log-transformed) testosterone concentrations were then standardised to have a variance of 1 by dividing the values by the standard deviation after correcting for calf age, sex of previous calf, collection time and assay date. This model was then fitted to estimate sex-specific V_A, V_{YOB} and V_R along with all associated between-sex correlations for V_A and V_{YOB} (though note that a cross-sex V_R correlation does not exist), and was used in subsequent testing.

In order to test whether male and female testosterone concentrations had the same underlying genetic structure, a model where V_A was allowed to vary between the sexes was compared to one where both sexes were constrained to be the same (and the correlation between them constrained to be 1), using LRTs which assumed a chi-squared distribution with 2 degrees of freedom.

4.5. Results

4.5.1. Heritability of neonatal testosterone concentration

The univariate model returned a h^2 estimate of 0.16 (± 0.06 SE) across both sexes, which was significantly greater than 0 (p=0.003; Table 4.2). Birth year accounted for an additional 4.3% of the variance observed (p=0.042; Table 4.2).

Table 4.2: *Components of variance in circulating calf testosterone concentrations from univariate model (854 calves from 17 years). See Table D. 1 for fixed effects.*

	Component ± SE	***p***	**Proportion of var. ± SE**
V_A	0.049 ± 0.020	0.003	0.160 ± 0.064
V_{YOB}	0.013 ± 0.008	0.042	0.043 ± 0.024
V_R	0.243 ± 0.021		0.797 ± 0.076

4.5.2. Cross-sex genetic correlation

When the sexes were allowed to have different genetic variances for calf testosterone, V_A appeared lower in males than in females (Table 4.3). Constraining this model to have equal V_A and a between-sex genetic correlation of 1 did not create a significantly worse model ($\chi^2_{(2)}$=0.060, p=0.971). This model did not, however, correct for the fact that males have greater variation around mean testosterone levels than females do (chapter 3.4), and so the model estimates could not be used to draw direct comparisons between the sexes.

Table 4.3: *Bivariate animal model estimating variance (diagonal), covariance (below diagonal) and correlation (above diagonal) components between female (n=434) and male (n=420) testosterone concentrations using unstandardized data, i.e.* ***with different sex-specific phenotypic variances****. Standard error (SE) estimates are in parentheses.*

V_A

	F	M
F	0.082 (0.033)	0.628 (0.399)
M	0.044 (0.027)	0.060 (0.044)

V_{YOB}

	F	M
F	0.012 (0.009)	0.986 (0.407)
M	0.015 (0.009)	0.019 (0.014)

V_R

	F	M
F	0.181 (0.029)	NA
M	NA	0.266 (0.041)

After standardising for sex-specific phenotypic variance, V_A (equivalent to h^2 in this model of standardised data) appeared lower in males (0.09 ± 0.12 SE) than females (0.30 ± 0.12 SE; Table 4.4), but the estimated genetic correlation was close to 1 (0.94 ± 0.65 SE). Constraining the model to have equal V_A and a between-sex correlation of 1, did not create a significantly worse model ($\chi^2_{(2)}$=2.178, p=0.336). This indicated that despite apparent differences, in this population V_A was not significantly different between sexes. The sexes therefore had a shared genetic source for calf testosterone concentration.

Table 4.4: *Bivariate animal model estimating variance (diagonal), covariance (below diagonal) and correlation (above diagonal) components between female (n=434) and male (n=420) testosterone concentrations* ***after being standardised for sex-specific variance****. SE estimates are in parentheses.*

V_A

	F	M
F	0.295 (0.121)	0.942 (0.648)
M	0.157 (0.083)	0.094 (0.115)

V_{YOB}

	F	M
F	0.039 (0.030)	0.998 (0.395)
M	0.050 (0.029)	0.064 (0.045)

V_R

	F	M
F	0.688 (0.109)	NA
M	NA	0.871 (0.119)

4.6. Discussion

Chapter 4 presents the first estimates of the heritability and cross-sex genetic correlation in testosterone concentrations within a wild mammal population. Analyses focused specifically on neonatal concentrations, which are under-represented in the literature, and made use of animal models rather than relying on parent-offspring or sibling analyses which have dominated estimates of testosterone heritability in the past. The results are discussed below.

4.6.1. Heritability of neonatal testosterone concentration

In this study population, neonatal testosterone has an estimated h^2 of 0.16 (± 0.06 SE) (Table 4.2). The statistical significance of this heritability estimate ($p=0.003$; Table 4.2) means that there is scope for neonatal testosterone concentrations to evolve should appropriate selection pressures exist. There is evidence of a relationship between these neonatal levels and calf survival within this population (chapter 3), however this is only apparent in a subset of individuals: males born to naive (first-time) mothers are less likely to survive to one year old if they had relatively high concentrations (but there was no effect in females or the sons of experienced mothers) (Figure 3.2). Consequently, selection on calf testosterone levels may be relatively weak. The implications of this, or how it relates to circulating hormone levels later in life (which may be under stronger selection), are not, however, fully understood.

The heritability of testosterone concentration was lower in this study than has been reported in other species (Table 4.1). Whilst exact heritability estimates are specific to the particular study individuals and their associated environmental conditions, there could also be other more general explanations for the differences between studies. Firstly, testosterone may reflect species differences, although no other estimates of testosterone heritability have yet been reported in red deer to allow comparison. Secondly, this study examined levels of testosterone in early life whilst most others focus on adult concentrations (see Table 4.1). Age-related variation in the heritability of testosterone concentrations has previously been shown in snakes (King et al. 2004), and in humans (Ring et al. 2005 and references therein), where heritability estimates ranged from 0 in new-born babies (Sakai et al. 1992, Caramaschi et al. 2012) to ≤0.66 in adolescents (Harris et al. 1998) and 0.19-0.91 in adults (see Table 4.1 for full list). The heritability of other traits has also been shown to vary across an individual's life due to differences in genetic and environmental effects (see Wilson et al. 2008 for discussion). Without further research into the ontogeny of within-population testosterone heritability however, further conclusions on this subject cannot be drawn. Thirdly, different methods of calculating heritability may also give rise to different estimates. For example, the parent-offspring or sibling relationships used in many previous studies may be inflated by shared environmental or maternal effects which can be more readily controlled in animal models (see Kruuk 2004 for details). Finally, most previous non-human studies have been lab based, whilst the red deer population in this study is completely wild. Heritability estimates in wild populations may be lower than those in captive populations because of exposure to more environmental variability (Charmantier and Garant 2005). A split family experiment in fall field crickets (*Gryllus pennsylvanicus*) has shown heritability estimates of wing dimorphism to be considerably lower amongst caged individuals raised in field (h^2= 0.21) than their caged relatives raised in the lab (h^2= 0.71) (Roff and Simons 1997).

In addition to the effects of V_A, there was also a small but significant calf cohort effect (Table 4.2) indicating the importance of environmental effects during foetal development/early life in determining individual testosterone levels. It was surprising that maternal identity did not also account for any variance, as strong

maternal effects have been associated with other birth traits within this population (Kruuk and Hadfield 2007).

4.6.2. Cross-sex genetic correlation

V_A appeared to be somewhat higher in females than in males, which might allow for the independent evolution of female calf testosterone concentrations if a response to selection were based on genes that did not influence male concentrations. Despite this, there was a high cross-sex genetic correlation (0.94 ± 0.65 SE), and a model in which the sexes were allowed to have different levels of genetic variance was no better than one in which the sex-specific variances were constrained to be equal. Overall, this suggests that testosterone concentrations are determined by the same underlying genetics in both sexes. This concurs with cross-sex genetic variance analyses in human adolescent twins (Hoekstra et al. 2006), and with inferences drawn in previous cross-sex testosterone studies in adults of other taxa (e.g. Ketterson et al. 2005, Moller et al. 2005, Mank 2007, Schroderus et al. 2010), suggesting that testosterone concentrations are determined by the same genes in both sexes.

The significantly non-zero heritability estimate means that there is scope for circulating neonatal testosterone concentrations to evolve under appropriate selection pressures. Whilst the high cross-sex genetic correlation means that the level of testosterone production could not evolve independently in the two sexes, there is evidence that conflict over optimal levels of testosterone for males and females could be resolved through differential gene expression and the independent evolution of testosterone *usage* between the sexes (e.g. Burns et al. 2014, Peterson et al. 2014). Given that neonatal testosterone levels were only linked to survival in a subset of animals (firstborn males; chapter 3), however, selection on this trait is likely to be relatively weak. Whilst chapter 3 gives no indication of sexually-antagonistic selection via calf survival on neonatal levels, sexually antagonistic selection has been recorded in adults of other species (Moller et al. 2005, Schroderus et al. 2010, Mills et al. 2012). There is also evidence of differences in sexual optima amongst neonates of other species (e.g. rats: Pfaff and Zigmond 1971, Christensen

and Gorski 1978). For example high testosterone concentrations are shown to be necessary for the masculinisation and defeminisation of male traits in rats (Roffi et al. 1987, Matuszczyk et al. 1990, Foecking et al. 2005). Experimentally suppressing testosterone in male rats or elevating it in females can have detrimental life-long implications to sex-linked behaviours, including mounting and sexual receptivity (Pfaff and Zigmond 1971, McDonald and Doughty 1972, Christensen and Gorski 1978, Corbier et al. 1983, Motelica-Heino et al. 1993), which may indicate sexual antagonism for neonatal as well as adult testosterone concentrations. These studies, however, do not explore the subtle among-individual variation upon which selection would act, and so the presence of sexually antagonistic selection pressures in neonatal testosterone concentrations cannot yet be confirmed. Whilst this chapter indicated a positive genetic correlation between male and female calf testosterone concentrations, no evidence exists at present to suggest that this has led to any between-sex conflict. It is not known how circulating neonatal testosterone levels in any taxa relate to their circulating levels later in life (although see chapters 5 & 6 for analysis of repeatability of FAM levels). Thus, to date, the strength of selection and the degree of sexual antagonism for this trait remain unresolved, and predicting its likely evolutionary dynamics requires further research.

4.6.3. Conclusion

This study was one of the first to use animal models in estimations of hormone heritability (see also: Schroderus et al. 2010), and the first in a wild mammal population. Circulating calf testosterone levels were found to be both heritable and to have similar genetic architecture in both sexes. This indicates that there is scope for this trait to evolve should the selection pressures exist, however the sexes cannot evolve independently. This may have important implications if the sexes have divergent optima which could constrain the evolution of this trait.

5 | Male steroids & reproductive effort

Cortisol but not testosterone is repeatable and varies with reproductive effort in wild red deer stags[3]

[3] (Pavitt et al. In review)

5.1. Summary

Although it is known that hormone concentrations vary considerably between individuals within a population, how they change across time and how they relate to an individual's reproductive effort remains poorly quantified in wild animals. Using faecal samples collected from wild red deer stags, this study first explored how FAM and FCM levels varied with both age and season, before quantifying the repeatability of these hormone groups among individuals. Finally, after correcting for other causes of variation I tested the relationship between these two hormone groups and a male's reproductive effort.

Results from these analyses showed clear temporal trends in concentrations of both hormones, including peak hormone levels during the autumn rutting reason. FAM (but not FCM) levels also showed significant among-individual variation across the 10-year sampling time period, which accounted for 35% of the trait's phenotypic variance after correcting for the age and season effects. Finally, I showed FCM to be positively correlated with male effort during the rut, both among and within individuals, which may be driven by the effect of social rank on both traits.

5.2. Introduction

Red deer stags exhibit considerable variation in their reproductive output, both within and between individuals over time (Nussey et al. 2009). Whilst much of the annual variation in this trait can be explained by factors linked to fighting ability such as age and body size (Clutton-Brock et al. 1982, Nussey et al. 2009), how hormones affect short term reproductive traits isn't well understood. In chapter 5, I looked at whether hormone concentrations could account for any additional individual variation by looking at their relationships with male reproductive behaviour during the annual rut.

5.2.1. Androgens

Androgen concentrations do not remain consistent across a male's lifetime, but often vary within and between years in association with behavioural changes

(Wingfield et al. 1990, Book et al. 2001 and references therein, Lynch et al. 2002). Within a year, temporal variation in testosterone concentrations often correlate with reproductive cycles and associated changes in male-male conflict (Wingfield et al. 1990, Lynch et al. 2002). Seasonally-reproducing, polygynous species such as red deer might be expected to show peak testosterone levels during the reproductive period, corresponding with the height of male aggression (e.g. Lincoln et al. 1972, Suttie et al. 1984, Bubenik and Schams 1986, Pereira et al. 2005). In the Rum red deer study population, maximum androgen levels would therefore be expected during the September-November rut when males compete with one another for access to oestrus females (Lincoln 1971). Where there is substantial age-related variation in reproductive effort, androgen concentrations might also be expected to vary with age (Book et al. 2001 and references therein). Red deer stags show considerable variation in reproductive effort and output across their lifetime (Nussey et al. 2009), with stags in their reproductive prime engaging in more aggressive encounters than both younger and older individuals (Clutton-Brock et al. 1979). They might, therefore, also be expected to exhibit higher testosterone concentrations overall (as has been shown in other deer species: Bubenik and Schams 1986).

Links between testosterone concentrations and male fitness-related traits are well established in several taxa (see reviews by Wingfield et al. 2001, Hau 2007), including red deer where high testosterone levels are associated with enhanced fitness-linked traits (Lincoln et al. 1972, Malo et al. 2009). Less is known, however about the relationship that testosterone may have with direct behavioural investment in reproduction. Red deer stags exhibit dominance hierarchies throughout the year (Lincoln et al. 1972, Bartos et al. 2010), which determine their access to females during the breeding season (Clutton-Brock et al. 1982) and thus their chances of siring offspring in that year. Given the positive relationships between testosterone, aggression and social rank (Muller and Wrangham 2004a, Reed et al. 2006, Bartos et al. 2010), testosterone levels might therefore also be expected to show a positive relationship with the size or length of time harems are held for (i.e. reproductive effort), and through that, a stag's annual reproductive success (Gibson and Guinness 1980, Appleby 1982, Pemberton et al. 1992). Similar results have been shown in an experimentally manipulated population of dark-eyed

junco, where the increased reproductive output in high testosterone males was attributed to testosterone-linked behavioural changes (Reed et al. 2006). Un-manipulated studies, however, are still needed to test whether this relationship remains true when considering subtler natural variation at the individual level in wild populations.

5.2.2. Glucocorticoids

Expectations for cortisol, the primary glucocorticoid in red deer (Ingram et al. 1999), and the second hormone considered here, are somewhat more complex. Whilst levels of this hormone are generally highest when animals are exposed to unpredictable or uncontrollable stressors (Greenberg et al. 2002), there is considerable variation in baseline levels between individuals. Where observed, circannual cycles in cortisol concentration are likely to reflect seasonal variation in stressors, such as challenging climatic conditions (e.g. low temperature: Huber et al. 2003a) or social instability (e.g. male conflict during the breeding season: Strier et al. 1999, Lynch et al. 2002). Males investing greater effort in reproduction might also have higher levels of cortisol if that effort is associated with energetic or physiological costs (e.g. the Cort-Adaptation Hypothesis: Bonier et al. 2009a, Bonier et al. 2009b), or if this is an adaptive response which enables them to maximise their fitness in unpredictable environments (Boonstra 2013). Given that a stag's energetic investment in reproduction peaks during middle-age in red deer (Clutton-Brock et al. 1979, Nussey et al. 2009), stags might also be expected to have high levels of cortisol during their reproductive prime (Nussey et al. 2009). Evidence, however, suggests that this might be confounded by the physiological effects of aging on circulating cortisol levels, with different study systems showing different physiological responses (e.g. Sapolsky et al. 1986, Otte et al. 2005, Heidinger et al. 2010). Circulating cortisol levels may increase with age due to desensitisation of the cortisol feedback loop (Sapolsky et al. 1986, Sapolsky 1991, van Cauter et al. 1996), or decrease with age (Jessop and Hamann 2005, Otte et al. 2005, Heidinger et al. 2010) because investment in present reproduction (which may be negatively affected by high cortisol levels: Liptrap 1993, Dobson and Smith 1995) is favoured over investment in longer-term survival (Wingfield and Sapolsky 2003).

Similarly, it is also difficult to predict the direction of associations between cortisol levels and social dominance. Red deer stags exhibit dominance hierarchies throughout the year (Lincoln et al. 1972, Appleby 1982, Clutton-Brock et al. 1982, Bartos et al. 2010), with higher ranking individuals monopolising resources such as high quality food (Lincoln et al. 1972) and reproductive opportunities (Clutton-Brock et al. 1982). If the maintenance of rank and resource access involves greater aggression and energetic investment (Lincoln et al. 1972, Clutton-Brock et al. 1979), then dominant males might also be expected to have higher cortisol levels as a result (e.g. Muller and Wrangham 2004b). This scenario would also predict positive associations between androgens and cortisol. The alternative hypothesis is that baseline glucocorticoid levels would be highest in animals with *lower* relative fitness (e.g. the Cort-Fitness Hypothesis: Bonier et al. 2009a), due, for example, to poorer quality or suppressed reproductive systems (Liptrap 1993, Dobson and Smith 1995). If high cortisol concentrations were linked to reduced quality and fitness, then individuals with high levels might also be expected to die at a younger age, leading to a population-level decline in cortisol concentrations amongst older age classes (van de Pol and Verhulst 2006).

This chapter (a) quantifies individual variation in faecal androgen and cortisol metabolites, (b) explores how concentrations of these hormones are affected by age and season, and (c) tests the relationships between concentrations of these hormones and an index of male reproductive effort (cumulative measure of daily harem size) during the breeding season.

5.3. Methods

5.3.1. Faecal hormone metabolite analysis

Faecal samples (n=194) were collected from 73 individually identifiable wild red deer stags in the North Block study of the Isle of Rum NNR, Scotland between 2004 and 2013 (see Figure 2.1a for distribution of repeat sampling between individuals). Stags were either born in the study area (n=53 males) or were visiting stags born in other parts of the island (n=20 males). Full details of faecal sample collection are

outlined in chapter 2.1.2. Hormones were then extracted from faecal samples (see chapter 2.4), and concentrations of both FAM and FCM were measured using 17-oxo-androgen (see chapter 2.5 for assay validation) and 11-oxoetiocholanolone (Huber et al. 2003b) EIAs respectively (see chapter 2.6 & 2.7).

5.3.2. Reproductive effort

Red deer are a polygynous species in which males compete for harems of females during the breeding season (Clutton-Brock et al. 1982). In this study, an index of male reproductive effort was measured by a stag's total number of "hind-days held" in a given year. "Hind-days held" was defined as the sum of a stag's daily harem size across the rutting season (15th September- 15th November) for a given year, based on daily censuses taken during this period. Censuses recorded male-female associations and used proximity and behaviours to assign females to a male's harem. By combining a stag's harem size on a particular day and the number of days he held a harem for, this index of reproductive effort is a good measure of total investment in a given year, and is closely linked to both social rank and reproductive success in stags (Gibson and Guinness 1980, Appleby 1982, Pemberton et al. 1992). These analyses used records of harem holding collected between 1971 and 2013, comprising of 2833 measures of reproductive effort from 815 stags. Only stags with at least 1 hind-day held were included in this analysis because the data could not distinguish between those who held no harem in a given year and those who held a harem outside of the study area.

5.4. Statistical analyses

A multivariate ("multi-response") mixed model was fitted to the data in ASReml-R 3.0.3 (package: asreml, Butler 2009) to explore potential causes of variation in, and covariances between, FAM, FCM and male reproductive effort. All three measures were log-transformed to normalise residuals.

Although FAM and FCM concentrations were only available for a subset (2004-2013) of the individuals for whom measures of reproductive effort exist, all individuals with observations of reproductive effort (1971-2013) were included in

the multivariate models, with missing values for FAM and FCM where necessary. Inclusion of these individuals improves the accuracy of estimation of the variance components associated with reproductive effort. Further, improved information on the distribution of reproductive effort both improves the accuracy and reduces the uncertainty (SE) of estimation of any covariance between reproductive effort and FAM or FCM. As outlined below, these covariances were estimated at both among-individual and within-individual (i.e. residual) levels.

From the original 194 faecal samples collected from 73 stags, 19 FAM and 16 FCM measures were removed due to low repeatability of concentrations measured between duplicates (CV>10%). A further 34 FAM measures were removed because they fell below the LOD (removal of these 34 FAM measured did not affect the results of the model, see Table E. 1 & Table E. 2). A total of 141 FAM measures (from 66 stags), 178 FCM measures (from 67 stags), along with 2833 measures of reproductive effort (from 815 stags), were therefore analysed. Of the measures included in this analysis, 105 measures of reproductive effort had corresponding FAM concentrations, and 138 had corresponding FCM concentrations. A further 33 FAM and 43 FCM concentrations were also included for stags which were either below rutting age (<4 years old; FAM: n=23 from 13 stags; FCM: n=32 from 12 stags), or did not hold a harem within the study area in the year of sample (FAM: n=10 from 5 stags; FCM: n=11 from 5 stags). No value for reproductive effort was assigned to any of these samples because it was unknown whether stags of reproductive age held harems outside the study area. Where stags had repeat measures of hormone concentration in a given year, the sample collected closest to the start of their harem holding period was associated with their measure of reproductive effort for that year. This allowed estimation of the residual covariance between reproduction effort and hormone concentration. These models therefore included 71 FAM concentrations and 82 FCM concentrations that were associated with a corresponding measure of reproductive effort, although all measures of hormone concentrations were included in the analyses (FAM: n=141; FCM: n=178).

5.4.1. Causes of hormone variation

Models contained the following fixed effects for all or most of the response traits:

Age: A continuous variable measured in years, and fitted for all three response variables. The quadratic term for age was also fitted FAM and reproductive effort. A quadratic relationship between age and FCM levels was tested but this was not significant (p=0.541) and so was excluded from the final analyses. The quadratic term for age was tested because a number of male reproductive traits are known to have a quadratic relationship with age in this population (Nussey et al. 2009).

Age at last sampling: A continuous variable measured in years, and fitted for both hormone concentrations. This was used to test for the selective disappearance of individuals with particular hormone phenotypes, allowing within-individual and population-level changes to be distinguished between (van de Pol and Verhulst 2006).

Sample month: An 11-level factor for the month in which the faecal sample was collected (January-November), fitted for both hormone concentrations.

Assay date: A 7-level factor for the date of faecal hormone assay, fitted for both hormone concentrations. This was included because previous studies have found assay date to account for significant variation amongst samples, possibly due to fluctuations in laboratory temperature (e.g. Graham et al. 2010; see also chapter 3 for discussion).

Time of sample collection (range: 09:15-21:10), and time from sample collection to freezing (range: 2-391 minutes) were also tested for effects on levels of FAM and FCM, as both have been shown to affect hormone concentrations (Suttie et al. 1992, Ingram et al. 1999, Mostl et al. 1999). There was not, however, a significant effect of either collection time (FAM: p=0.836; FCM: p=0.801) or time to freezing (FAM: p=0.780; FCM: p=0.891), and so both were excluded from the final model. Fixed effects were tested for significance using incremental Wald tests, and the optimal model was accepted when all remaining fixed effects were significant at $p<0.05$.

5.4.2. Hormones & reproductive effort

Individual identity (n=815), year of sampling (n=42), and unexplained residual effects were also fitted as random effects for all three traits in this model. After comparing nested models fitted with and without year of sampling, this random effect was excluded from the final model because it did not significantly affect any of the three traits (FAM: p=0.945; FCM: p=0.720; reproductive effort: p=0.492; Table E. 3). The repeatability of all three traits was estimated as the proportion of that trait's overall phenotypic variance that was accounted for by individual identity (i.e. among-individual differences).

After testing the variances associated with individual identity and residual effects, covariances between the respective random effects were also fitted to explore relationships between the three traits at both individual and residual levels. In order to test the significance of all covariances, LRTs were used to compare the full model with models where each particular covariance was fitted to 0 in turn. The LRT assumed the difference in the likelihood of the two models was a chi-squared distribution with 1 degree of freedom. Because no individual-level variation was found in FAM concentrations when fitting a multivariate model with just variance components (p=0.664, Table E. 3), the among-individual covariances between FAM and both FCM and reproductive effort were fixed at 0 in the final model. This model was not a significantly worse fit to the data than a model in which these covariances were estimated (LRT: $X^2_{(2)}$=0.451; p=0.637), but is more statistically justified than estimating the covariance between two parameters when there is no robust statistical evidence of any significant variance in one of them. In the final model, therefore, the only testable (i.e. non-zero) among-individual covariance was between FCM and reproductive effort.

5.5. Results

Both faecal androgen and cortisol metabolite concentrations varied substantially between samples. Concentrations of FAM ranged from 2.66-17216.31 ng/g faeces (mean concentration: 449.20 ng/g faeces ± 151.79 SE), and FCM from 5.29-680.97 ng/g faeces (mean concentration: 61.53 ng/g faeces ± 6.28 SE). Measures of

reproductive effort also varied considerably, ranging from 1-646 hind-days held (mean: 56.2 hind-days held ± 1.5 SE).

5.5.1. Seasonal & age effects

Concentrations of both hormones showed significant variation with month ($p<0.001$; Table 5.1; Figure 5.1). FAM levels peaked in September, decreased through October and overall remained low for the rest of the year (Figure 5.1). FCM concentrations also increased during the autumn period (with peak concentrations September-October), but showed an additional peak in February-March (Figure 5.1). FAM, FCM and reproductive effort also varied significantly with a stag's age (FAM: $p<0.001$; FCM: $p=0.012$; reproductive effort: $p<0.001$; Table 5.1; Figure 5.2 & Figure 5.3), however age at final sampling did not significantly improve the model when considered for either hormone (FAM: $p=0.777$; FCM: $p=0.864$; Table 5.1). FAM concentrations increased with age until around 7-10 years old, after which they began to decline (Figure 5.2a). In accordance with previous studies of this population (Nussey et al. 2009), levels of reproductive effort also peaked around 8-11 years old (Figure 5.3). By contrast the relationship between FCM and age was linear, with older individuals having higher concentrations (Figure 5.2b). In agreement with previous studies (Graham et al. 2010; see also chapter 3), both FAM and FCM varied with assay date.

Table 5.1: *Multivariate mixed effects model estimating the main effects of extrinsic factors on individual-level variation in stag (a) FAM, (b) FCM and (c) reproductive effort.*

FIXED EFFECTS	**(a) FAM (141)**			**(b) FCM (178)**			**(c) Repro. effort (2833)**		
	Est. SE	*p*		Est	*p*		Est	*p*	
Age	**0.117 ± 0.061**	**0.032**	*	**0.058 ± 0.031**	**0.012**	*	**0.174 ± 0.009**	**<0.001**	***
Age^2	**-0.047 ± 0.010**	**<0.001**	***	-	-		**-0.038 ± 0.002**	**<0.001**	***
Age at final sample	-0.019 ± 0.060	0.777		-0.005 ± 0.032	0.864		-	-	
February [a]	-0.251 ± 0.656			0.371 ± 0.311			-		
March [a]	-0.925 ± 0.715			0.393 ± 0.320			-		
April [a]	-0.386 ± 0.653			-0.051 ± 0.310			-		
May [a]	-0.191 ± 1.045			0.224 ± 0.410			-		
June [a]	-1.121 ± 1.366	**<0.001**	***	-0.179 ± 0.664	**<0.001**	***	-	-	
July [a]	-0.342 ± 0.735			-0.088 ± 0.339			-		
August [a]	0.312 ± 0.532			0.787 ± 0.250			-		
September [a]	1.745 ± 0.504			0.803 ± 0.245			-		
October [a]	1.054 ± 0.538			0.801 ± 0.252			-		
November [a]	-0.157 ± 0.648			0.458 ± 0.308			-		
Assay date	***7 estimates***	**<0.001**	***	***7 estimates***	**<0.001**	***	-	-	

[a] Estimates for month are relative to estimates of January

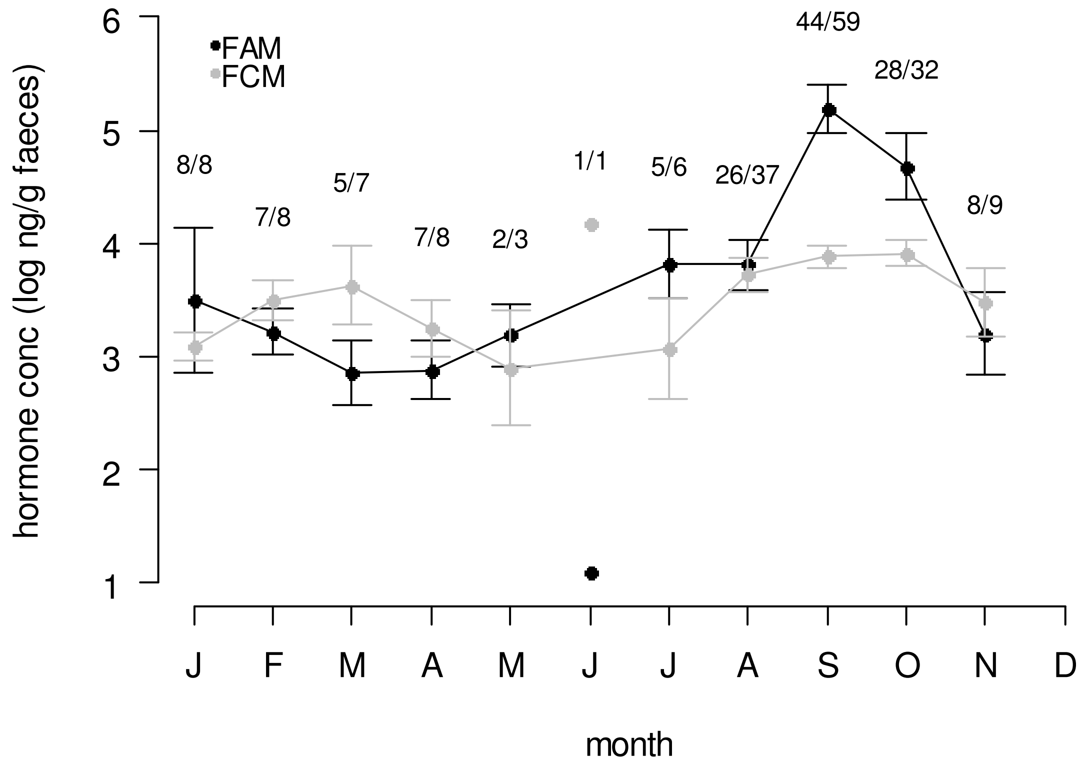


Figure 5.1: *Variation in log transformed FAM (black) and FCM (grey) concentrations with month. Points represent monthly means ± SE. Numbers represent monthly sample sizes for FAM/ FCM respectively. Only one sample was collected in June and so no estimate of error was possible.*

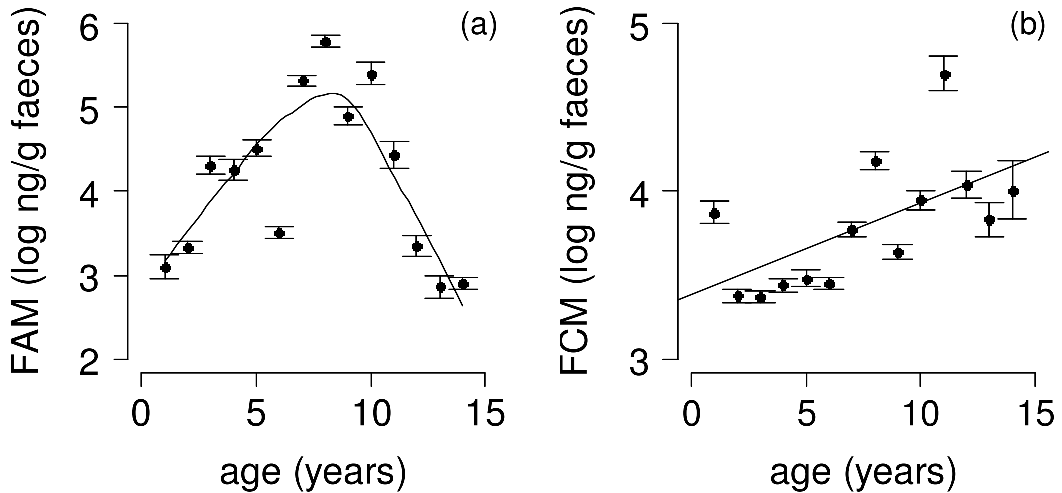


Figure 5.2: *Variation in log-transformed (a) FAM and (b) FCM with a stag's age. Points represent means ± SE, and the smooth lines were fitted from regressions of log-transformed hormone concentrations against age.*

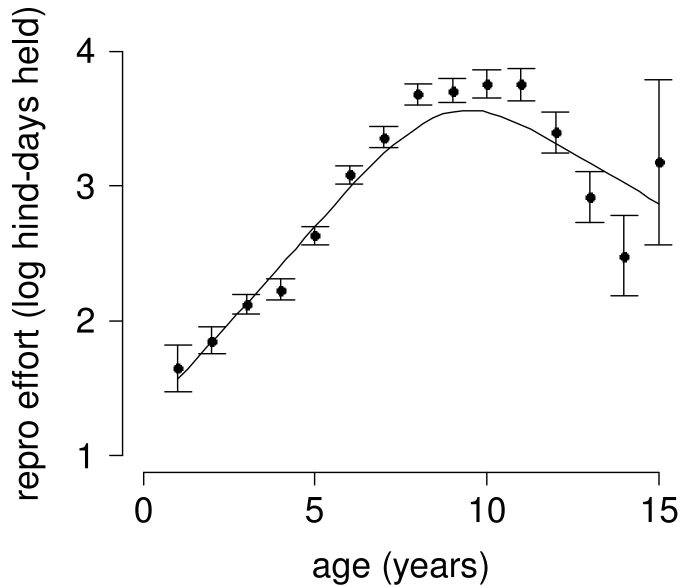


Figure 5.3: *Variation in log transformed reproductive effort with age. Points represent means ± SE, and the smooth line was fitted from a regression of log-transformed reproductive effort against age.*

5.5.2. Variance components

FAM levels were not repeatable among individuals (p=0.719; Table 5.2a), with differences between stags only accounting for 3% (0.03 ± 0.10 SE) of the overall variance observed in this trait. In contrast FCM and reproductive effort varied significantly both at the among- and within-individual levels (p<0.005; Table 5.2). FCM has a repeatability of 0.35 ± 0.10 SE (i.e. individual identity accounted for around 35% of the variation seen in this trait after correcting for the fixed effects), and reproductive effort had a repeatability estimate of 0.35 ± 0.02 SE.

Table 5.2: *Multivariate mixed effects model estimating variances (diagonal), covariances (below diagonal), and correlations (above diagonal) for FAM, FCM and reproductive effort at (a) among-individual and (b) residual within-individual levels (SE in parentheses). Shaded cells indicate values that were fixed and not allowed to vary. Statistically significant variances and covariances are in bold. See Table 5.1 for the fixed effects from this model.*

(a) among-individual

	FAM	FCM	Effort
FAM	0.049 (0.154) X^2=0.065 *p*=0.719	0	0
FCM	0	**0.184** **(0.068)** **X^2=4.245** ***p*=0.004**	0.577 (0.182)
Effort	0	**0.208** **(0.080)** **X^2=3.067** ***p*=0.013**	**0.711** **(0.062)** **X^2=113.574** ***p*<0.001**

(b) within-individual

	FAM	FCM	Effort
FAM	**1.481** **(0.230)** **X^2=256.044** ***p*<0.001**	-0.011 (0.108)	-0.215 (0.135)
FCM	-0.008 (0.078) X^2=0.006 *p*=0.910	**0.347** **(0.048)** **X^2=60.068** ***p*<0.001**	0.268 (0.125)
Effort	-0.300 (0.191) X^2=1.139 *p*=0.131	**0.192** **(0.091)** **X^2=1.876** ***p*=0.049**	**1.317** **(0.041)** **X^2=9579.851** ***p*<0.001**

5.5.3. Hormones & reproductive effort

Stags with higher reproductive effort were also likely to have higher FCM concentrations (see Figure 5.4 for overall phenotypic relationship between these two variables). This positive correlation between reproductive effort and FCM was found both at the among-individual (LRT: p=0.013; Table 5.2a), and within-individual, or residual, (LRT: p=0.049; Table 5.2b) levels. Given that FAM concentrations were not repeatable amongst stags, no among-individual covariance between FAM and either FCM or reproductive effort was estimated (see Table 5.2). There were, however, non-significant negative covariances within individuals between FAM, and both FCM (LRT: p=0.910; Table 5.2b) and reproductive effort (LRT: p=0.131; Table 5.2b).

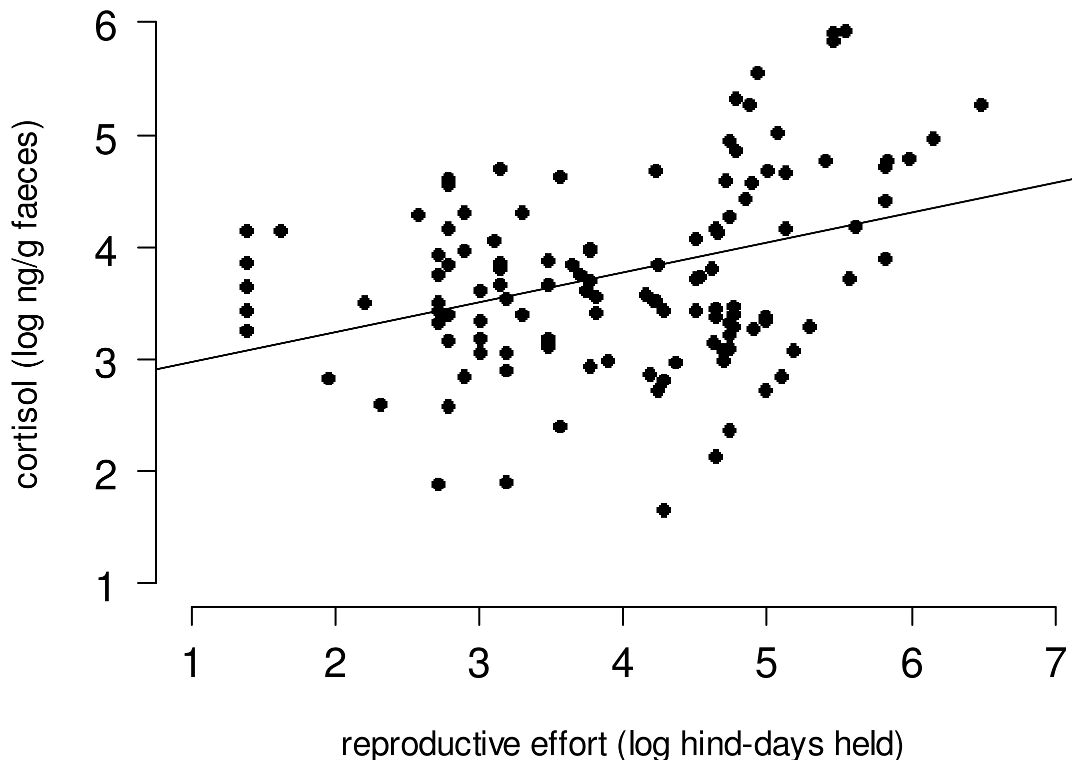


Figure 5.4: *The phenotypic relationship between log-transformed FCM concentrations and log-transformed reproductive effort (n=135 observations from 50 stags). Figure shows the raw data, with a fitted line from the regression of log-transformed FCM against log-transformed reproductive effort.*

5.6. Discussion

Chapter 5 shows clear age and season related trends in levels of both FAM and FCM. There was also a significant positive relationship between a stag's FCM levels and an index of their reproductive effort during the corresponding breeding season at both the among- and within-individual levels. This analysis is amongst the first to test assumptions about the relationships between FAM and FCM concentrations and reproductive effort in the wild.

5.6.1. Androgens

In accordance with expectations, FAM levels were highest in the build up to and during the reproductive season (August-October), and in prime-aged stags (aged 7-10 years old). Testosterone is known to regulate the expression of both reproductive and aggressive behaviours in red deer (Lincoln et al. 1972, Fletcher 1978); rutting behaviour, for example, can be eliminated by castrating a red deer stag, and restored through testosterone implants (Lincoln et al. 1972). It was therefore not surprising to observe maximum FAM levels during the rut when inter-male aggression is greatest, and at the age when male annual reproductive performance, and thus presumably agonistic interactions between competing males, peaks (Nussey et al. 2009).

In contrast to several previous wild studies (Pelletier et al. 2003, Kralj-Fisher et al. 2007, While et al. 2010), the analyses presented in this chapter showed no evidence of among-individual variance in FAM levels. It is worth noting, however, that these previous studies were either based on much smaller sample sizes (Kralj-Fisher et al. 2007, While et al. 2010), or considered repeatability within the shorter time-periods of days (Pelletier et al. 2003) or months (Kralj-Fisher et al. 2007, While et al. 2010), whilst this chapter utilised samples collected over several years. Given that sample year also explained no variance (see chapter 5.4), this lack of among-individual FAM repeatability cannot be attributed to annual variation above the effects of age. Whilst a positive relationship between FAM and reproductive effort was expected (see reviews by Wingfield et al. 2001, Hau 2007), this was not found to be the case here. Instead there was a non-significant *negative* trend at the within-individual level

(p=0.131; Table 5.2b), which is possibly more in concurrence with negative relationships between testosterone levels and dominance seen in populations during periods of social hierarchical instability (Bartos et al. 2010).

5.6.2. Glucocorticoids

This study identified two peaks in FCM concentration across the year, coinciding with periods of high environmental or physiological stress; one during the late winter (peaking in March), and a second one during the early autumn. The first peak is similar to previous findings in captive red deer (Huber et al. 2003a), and falls towards the end of winter. Winter is known to be energetically challenging for the deer on Rum, particularly males, with limited food availability and high mortality rates (Clutton-Brock et al. 1982). The second peak in FCM coincides with the rutting season, and may be the result of increased agonistic interactions between stags competing for females (see Romero and Butler 2007 for discussion of the stress-response). Elevated cortisol levels during the reproductive season have been reported in males of other polygynous species (Strier et al. 1999, Lynch et al. 2002), although this has not previously been found when analysing seasonal variation in red deer (Ingram et al. 1999, Huber et al. 2003a). This lack of consensus with other deer studies may arise because previous work has focussed on captive deer which may not have been exposed to the same conditions, behaviours or social interactions as those in the wild.

In concurrence with previous rat (see Sapolsky 1991 for review) and human (Halbreich et al. 1984, van Cauter et al. 1996) studies, cortisol concentrations increased linearly with age in this population. Laboratory experiments in rats have shown older individuals to take longer to return to baseline levels after a stressor, leading to prolonged periods of cortisol hyper-secretion (Sapolsky et al. 1984, 1986). The age-related increase in FCM levels observed in this chapter appeared to be a consequence of within-individual change (rather than change at the population level), as age at final sampling had no effect on FCM levels. This suggests that the observed age-related variation did not reflect the selective disappearance of particular hormone phenotypes with age (van de Pol and Verhulst 2006). After

accounting for age and sample month, FCM levels were also repeatable among stags. Overall, among-individual differences (i.e. the among-individual variance for FCM; Table 5.2a) explained ~35% of the total phenotypic variance of this trait (i.e. the sum of all FCM variance components in Table 5.2) after correcting for the fixed effects. Stags with relatively high FCM concentrations at one sampling point also had relatively high FCM concentrations at other sampling points (and *vice versa* for low FCM stags). This concurs with previous findings of repeatability in glucocorticoid metabolite concentrations across a period of several months in wild greylag geese (*Anser anser*) (Kralj-Fisher et al. 2007), although this chapter showed among-individual variance to remain across several years.

In this study, stags investing the greatest effort in reproduction (in terms of hind-days held) were also more likely to have high baseline FCM concentrations at both the among- and within-individual levels. In red deer, resources such as reproductive opportunity (Clutton-Brock et al. 1982) and high quality food (Lincoln et al. 1972, Appleby 1980) are monopolised by socially dominant stags. Stags compete throughout the year for access to these resources, with high ranking individuals involved in more agonistic and aggressive interactions as a result (Lincoln et al. 1972, Clutton-Brock et al. 1982). Indeed, experimental studies show that reducing aggression through castration causes stags to drop in social rank (Lincoln et al. 1972). Research also suggests that high dominance is conserved across the year, with stags who dominate in bachelor herds (i.e. male groups outside of the rut), maintaining their high rank in the subsequent rutting season (Clutton-Brock et al. 1982). Given the positive relationship between aggression and glucocorticoid levels observed in other systems (e.g. Muller and Wrangham 2004b), results from this study support the hypothesis that whilst social dominance enables a high investment in reproduction, it also has associated behaviours (such as agonistic interactions) which lead to corresponding high levels of FCM. This relationship can also be seen within individuals (Figure 5.2b): stags had higher FCM levels in years when they invested more reproductive effort than in years when they invested less. Whilst results from this study cannot be used to draw conclusions about the longer-term associations between cortisol and fitness beyond that of a single year, these

results do *not* support the hypothesis that cortisol will negatively influence a stag's reproductive effort within the year of sampling.

5.6.3. Conclusion

In summary, both FAM and FCM concentrations varied with age, and showed pronounced seasonal cycles, with both hormones peaking during the rutting season. Only FCM concentrations were repeatable among individuals: after correcting for age- and season-related variation, FAM concentrations showed no among-individual variance. Stags investing more effort during the rut (i.e. more hind-days held) had higher cortisol concentrations than those investing less effort. Given that stags with high measures of reproductive effort tend to be more dominant, this relationship with FCM may be the consequence of more aggressive encounters and effort invested in maintaining their dominance status. Importantly, these results also show that high baseline cortisol levels do *not* negatively affect a stag's reproductive effort, and thus opportunity, within the year of sample.

6 | Female steroids vary over time and with reproductive status[4]

[4] (Pavitt et al. In prep.)

6.1. Summary

Whilst androgen and glucocorticoid levels are known to vary within and among individuals in a population, the effects of mammalian gestation and lactation on a female's measurable hormone levels are less well understood. Using faecal samples collected from wild red deer hinds between 2001 and 2013, this study explored how FAM and FCM concentrations changed with age and season, and how individual differences may have arisen due to variation in reproductive status. Analyses then went on to explore whether levels of FAM and FCM during pregnancy could influence circulating testosterone concentrations in offspring.

This study showed clear temporal trends in concentrations of both hormone groups, including seasonal cycles, with peak hormone levels during late spring when females give birth. Pregnancy and lactation status also explained variation in hormone concentrations. Both FAM and FCM levels increased towards parturition, although this only affected FCM levels in older females. Whilst pregnancy status accounted for variation in hormone levels, neither FAM nor FCM levels during pregnancy appeared to influence circulated testosterone levels in the resulting calf. Lactating FCM levels were also higher when females suckled a male rather than a female calf, possibly due to the higher energetic costs of raising a son. This ultimately illustrates the importance of accounting for a female's life history and current reproductive status, as well as temporal variation, when examining individual differences in hormone levels.

6.2. Introduction

This chapter explores individual-level variation in concentrations of female androgens and glucocorticoids within the Rum red deer, and tested for associations with season and individual state. Reproduction is a large energetic investment for female red deer (Clutton-Brock et al. 1983, Loudon and Racey 1987, Catchpole et al. 2004) which will often have barren years between calves. This allows for investigation of natural endocrine differences between females in different reproductive states.

6.2.1. Androgens

Whilst androgens are commonly thought of as male hormones, they are also secreted in smaller concentrations by the ovaries and adrenal glands in females (Squires 2003). Amongst these, testosterone is the most widely studied, and whilst research typically focusses on its role in males it is now clear that testosterone levels also have important implications for fitness and life history traits in females (Taitt and Krebs 1982, Staub and DeBeer 1997, Clotfelter et al. 2004, Ketterson et al. 2005, Veiga and Polo 2008).

Female FAM concentrations may vary with both age and time of year in this population, as chapter 5 has already shown to be the case of stags in this population. Age-related changes may arise due to reduced functionality or responsiveness of the hypothalamic-pituitary-gonadal system (Flood et al. 1995), leading to the secretion of less testosterone, and an overall reduction in circulating concentrations of the hormone (e.g. Zumoff et al. 1980, Flood et al. 1995, Zumoff et al. 1995, Burger 2002, Davison et al. 2005). If high androgen concentrations are associated with reduced survival in females (e.g. Taitt and Krebs 1982), then mean levels might be expected to decline with age due to high androgen individuals dying younger (van de Pol and Verhulst 2006). Within a year, temporal variation in androgen levels often correlates with a number of factors, including seasonal variation in social interactions and physiological state. For example, in the ring-tailed lemur (*Lemus catta*), female androgen cycles show close correlation with those of males, with both peaking during the mating seasoning when the number of aggressive encounters is likely to be the highest (von Engelhardt et al. 2000). If this is true for females in the Rum red deer study population, androgen levels would be expected to peak during September-November to coincide with the rutting season when male androgen levels peak (Figure 5.1).

Numerous studies show testosterone to increase in pregnant females as they approach parturition (Demisch et al. 1968, Barkley et al. 1979, Bammann et al. 1980, Gaiani et al. 1984, Silberzahn et al. 1984, Musey et al. 1987, Michel et al. 2011). This has been attributed both to a direct increase in steroid production (e.g. late gestation

humans: Bammann et al. 1980, horses: Silberzahn et al. 1984), and to a reduction in the rate of hormone clearance (Bammann et al. 1980). If this were true for this study population, then females would be expected to have peak androgen levels during May-June when most give birth (Clutton-Brock et al. 1982). Lactation may also affect androgen concentrations, as testosterone levels are known to be lower in humans during lactation than non-lactation (Alder et al. 1986), possibly as a result of suppressed ovarian activity during this time (Howie et al. 1981).

6.2.2. Glucocorticoids

As with androgens, glucocorticoids also vary temporally with both age and season. Physiological aging has been linked to both increased and decreased glucocorticoid secretion (see chapter 5 for discussion of changes in red deer stags), although the effect may also be non-linear as females in this population reach their prime condition during middle age (around 9 years old: Nussey et al. 2006). If FCM levels are negatively associated with condition and reproductive performance, these middle-aged females might be expected to have lower levels because they are presumably best able to deal with the costs of reproduction (Clutton-Brock et al. 1983, Catchpole et al. 2004, Nussey et al. 2006).

Where they occur, seasonal glucocorticoid cycles are likely to be closely linked to seasonal variation in stressors such as climatic conditions (Huber et al. 2003a, Weingrill et al. 2004) or variation in reproductive state. Given that glucocorticoid concentrations increase towards the end of gestation across a range of mammals (McMillen et al. 1987, Atkinson and Waddell 1995, Cavigelli 1999, Obel et al. 2005), seasonal levels of cortisol might be expected to increase throughout spring and peak May-June (during the calving season) within this study population. Whilst concentrations likely increase as the developing foetus becomes more metabolically costly, other physiological changes might also have an effect. Higher glucocorticoids in late-term pregnancy may also arise due to increased sensitivity of the stress response (Scott et al. 1990, Obel et al. 2005), increased oestrogen secretion, which is positively correlated with cortisol levels (e.g. primates: Smith and Norman 1987,

Stavisky et al. 2003), and/or suppression of the day-night cortisol cycle (e.g. humans: Burke and Roulet 1970).

Despite the additional stress associated with early lactation, previous studies have found little difference between lactating and non-lactating females (Thoman et al. 1970, Altemus et al. 1995, Cook 1997) due to suppression of the acute stress response (Altemus et al. 1995, Rushen et al. 1995, Walker et al. 1995). Variation may, however, still arise between lactating individuals incurring different energetic costs (Cook 1997 and references therein, Hill et al. 2003, Ryan et al. 2012). For example, differences may arise depending on the sex of the calf, as sons are known to be more costly to mother than daughters in this population (see chapter 3.2.2 for discussion).

6.2.3. Maternal hormones & offspring testosterone

Variation in hormone levels between pregnant females might also influence foetal development, leading to differences in the concentrations of circulating hormones in offspring after birth. This could arise either as a consequence of direct hormone transfer between maternal and foetal bloodstreams, or as an indirect effect of the mother's internal and external environments. Whilst there is some evidence of testosterone moving from maternal to foetal bloodstreams in hyenas (Licht et al. 1998), in most studies the placenta acts as a barrier to testosterone transfer (Vreeburg et al. 1981, Slob et al. 1983, Sathishkumar et al. 2011, Sun et al. 2012). In the absence of direct transfer, maternal and offspring testosterone concentrations may, however, still be correlated if maternal levels are indicative of other factors influencing foetal development. For example, testosterone levels during pregnancy are associated with a number of offspring traits including birth weight (Carlsen et al. 2006)(Carlsen et al. 2006)(Carlsen et al. 2006)(Carlsen et al. 2006), behavioural masculinisation in female offspring (Clarke et al. 1976), and have also been proposed as a key factor in sex ratio determination (Grant 2007).

Such effects can also be seen in maternal stress studies where a stressful foetal environment may influence the development and maturation of individuals throughout their lives. For example, testosterone levels are often lower in male

neonatal rats when their mothers are exposed to stressful conditions during pregnancy, compared to those born to control mothers (Ward and Weisz 1980, 1984). This trend has been attributed to suppression of the masculinisation and defeminisation processes during development as a consequence of the mother's physiological response to the stressors (Ward and Weisz 1984). If high cortisol levels are indicative of an individual in a stressful environment (Moberg 2000, Romero and Butler 2007), then females with high cortisol levels might be expected to have offspring/sons with lower testosterone levels.

This chapter quantifies within- and among-individual variation in FAM and FCM concentrations female red deer, and examines the degree to which this variation can be accounted for by (a) age, (b) seasonal variation, and (c) reproductive status of that individual. After correcting for seasonal and state-dependent variation, the relationship between a mother's hormone levels during pregnancy and the levels of circulating testosterone in her resulting calf were then explored.

6.3. Methods

6.3.1. Faecal hormone metabolite analysis

Faecal samples (n=806) were collected from 200 wild red deer hinds in the North Block study of the Isle of Rum NNR, Scotland between 2001 and 2013 (see Figure 2.1b for distribution of repeat sampling between individuals). Full details of faecal sample collection are outlined in chapter 2.1.2. Hormones were then extracted from faecal samples (see chapter 2.4), and concentrations of both FAM and FCM were measured using 17-oxo-androgen (see chapter 2.5 for assay validation) and 11-oxoetiocholanolone (Huber et al. 2003b) (see chapter 2.6 & 2.7) EIAs respectively.

The subset of circulating calf testosterone levels used in this analysis (n=80) were collected between 2005 and 2012 (see chapter 2.1.1 for details of sample collection, and chapter 2.2 & 2.3 for extraction and assay methodology). Each calf included here was born to a mother who had a faecal sample collected when pregnant with that calf.

6.4. Statistical analyses

6.4.1. Causes of hormone variation

A multivariate ("multi-response") mixed model was fitted to the data in ASReml-R 3.0.3 (package: asreml, Butler 2009) to explore potential causes of variation in, and covariances between, female FAM and FCM levels, and the circulating testosterone levels in calves born to mothers sampled when pregnant. All hormone measures were log-transformed to normalise the residuals, and all continuous fixed effects were mean-centred. A total of 638 FAM measures (from 192 females) and 765 FCM measures (from 194 females) were included in this analysis after removing samples due to low repeatability between duplicates (CV>10%; FAM=11; FCM=15) or because they fell below the LOD (FAM=39; FCM=24). Removal of these <LOD measures did not affect the main outcomes of the model (see Table F. 1 & Table F. 2 for details).

Fixed effects

Models contained the following fixed effects for both faecal hormone groups:

Age: A continuous variable measured in years. The quadratic term for age was also tested but this was not significant for either FAM (p=0.082) or FCM (p=0.906), and so was excluded from the final analysis. The quadratic term for age was tested because female reproductive traits are known to have a quadratic relationship with age in this population (Nussey et al. 2009).

Age at last sampling: A continuous variable measured in years. This was used to test for the selective disappearance of individuals with particular hormone phenotypes, allowing within-individual and population-level changes to be distinguished between (van de Pol and Verhulst 2006).

Sample month: A 12-level factor for the month in which the faecal sample was collected.

Assay date: An 8-level factor for the date of faecal hormone assay. This was included because previous studies have found assay date to account for significant variation amongst samples, possibly due to fluctuations in laboratory temperatures (e.g. Graham et al. 2010; see also chapter 3 for discussion).

Pregnancy status: A 2-level factor for whether a female was (i) not pregnant or sampled in early pregnancy (defined as up to 118 days after estimated conception date) versus (ii) sampled during late pregnancy. There was no significant difference between samples from non-pregnant and early pregnancy females, and so these were combined into one factor level. Whether or not the female was pregnant at the time of defecation was based on records of calving date and estimated conception date (calculated as 235 days prior to the calving date: Clements et al. 2011).

Lactation status: A 3-level factor for whether a female was (i) suckling a male calf, (ii) suckling a female calf, or (iii) not lactating at the time of defecation. Females were considered to be lactating if they were sampled before April with a live calf (<1 year old) from the previous calving season. If females had not calved during the previous calving season, or had given birth to a calf that had died before the date of sampling, the female was considered to be "not lactating".

Time to freezing: A continuous variable measuring the time (in minutes) from faecal collection to freezing (in a -20°C field freezer). This was tested because hormone concentrations are known to change over relatively short periods of time in samples kept at ambient temperature (Mostl et al. 1999). Whilst collection and freezing times were not recorded for 203 samples, these samples were assigned the mean time to freezing (calculated from all samples which did have a measure) to avoid the loss of data. This gave the model greater statistical power and did not cause the trends in the main findings to change (see Table F. 3 for model output excluding samples with no recorded time to freezing).

Sample collection time was also tested because this has previously been shown to influence circulating steroids in red deer (Suttie et al. 1992, Ingram et al. 1999). Due to missing time data, this was fitted to models (i) including just samples with a collection time, and (ii) including all samples but where samples with no collection times were assigned the mean time. Collection time was non-significant for both FAM (i: $p=0.479$; ii: $p=0.519$) and FCM (i: $p=0.358$; ii: $p=0.449$) and so was not included in the final models.

Random effects

Individual identity, sampling year, and a residual (error) term were also included in the model as random effects. Inclusion of these random effects allowed estimation of the among-individual variance (variance due to individual identity), the among-year variance (variance due to year), and the within-individual (residual) variance. The use of multivariate models also allowed estimations of the covariance between traits at all three levels. Only the within-individual (residual) component explained significant variation, however, and so only within-individual covariances between FAM and FCM were estimated.

6.4.2. Maternal hormones & offspring testosterone

For females sampled when pregnant, circulating testosterone concentrations were also included for the resulting calves (n=80 calves). When females had repeat measures of hormone concentration taken when pregnant with a single calf (n=25 calves), only one sample was associated with the circulating testosterone measure of that calf. Two models were run to test for a covariance between maternal hormones and offspring testosterone differently, focussing on either (a) early (≤118 days) or (b) late (>118 days) pregnancy. When more than one sample was collected from a female pregnant with the same calf, the last FAM and FCM concentrations measured in the time period were associated with the measure of calf testosterone. The following fixed effects were included because they had previously been shown to explain substantial inter-individual variation in calf testosterone levels (chapter 3): calf age at capture; calf sex; sample collection time; and date of assay (see chapter 3.4.1 for details).

All fixed effects were tested for significance using incremental Wald tests, and the optimal model was accepted when all remaining fixed effects were significant at $p<0.05$. The variances and covariances were tested for statistical significance using LRTs (assuming a chi-squared distribution with 1 degree of freedom) comparing the full model with models where each (co)variance was fixed to 0 in turn.

6.4.3. Autocorrelation

To test that the lack of among-individual variance in FAM and FCM levels was not due to the between-sample covariance declining over time, univariate linear mixed effect models were also fitted individually to each hormone in R 3.1.1 (package: nlme, Pinheiro et al. 2014) with a correlation structure included. Where individuals were sampled more than once on the same day (n=12 instances), the mean value for that day was used. This enabled autocorrelation to be tested between samples collected on different days at the individual level. An ANOVA was used to test models with and without the correlation structure included, and found the models correcting for temporal autocorrelation between days were not significantly better than ones which did not (FAM: p=0.965; FCM: p=0.555; see Table F. 4 & Table F. 5 for model details). This indicated that the covariance between samples did not decline over time (i.e. no among-individual variance would be found even after restricting the data to samples collected close together), and therefore this was not the cause of the lack of among-individual variance. An autocorrelation structure was therefore not fitted to the final multivariate model.

6.5. Results

Levels of both FAM and FCM showed substantial variation between samples. FAM concentrations ranged from 1.02-311.70 ng/g faeces (mean: 22.93 ng/g faeces ± 1.16 SE) and FCM concentrations from 4.33-20720.00 ng/g faeces (mean: 106.35 ng/g faeces ± 31.87 SE).

6.5.1. Sampling effects

Variation in laboratory and collection conditions had small but significant effects on both hormone groups. Both FAM and FCM levels varied depending on the date they were assayed (p<0.001); FAM levels also increased with greater time at ambient (p=0.007), although this did not affect FCM levels (p=0.370; Table 6.1).

6.5.2. Seasonal & age effects

After accounting for variation due to assay date and the time from sample collection to freezing, concentrations of FCM but not FAM showed significant variation with age (FAM: p=0.220, FCM: p<0.001; Table 6.1), and with age of final sample (FAM: p=0.358, FCM: p=0.008; Table 6.1). FCM levels increased linearly with age (Table 6.1; Figure 6.1), but decreased with age at final sampling; this indicates that FCM increased with age *within* individuals, but that high FCM females were less likely to be sampled in the older age groups. Both hormones did, however, show similar patterns of monthly variation (p<0.001; Table 6.1; Figure 6.2). FAM levels spiked in May, whilst FCM increased through May to June and fell again in July to coincide with the calving season.

***Table 6.1**: Fixed effects from multivariate mixed effect model examining the main effects of season, age and reproductive state on inter-individual variation in female (a) FAM and (b) FCM concentrations. See Table F. 6 for the fixed effects of circulating neonatal testosterone levels from calves born to mothers sampled when pregnant with that calf.*

FIXED EFFECTS	(a) FAM (638)			(b) FCM (768)		
	Est. ± SE	*p*		Est. ± SE	*p*	
Age	0.014 ± 0.020	0.220		0.055 ± 0.016	<0.001	***
Age at final sample	-0.018 ± 0.019	0.358		**-0.042 ± 0.016**	**0.008**	**
February [a]	-0.037 ± 0.205			0.105 ± 0.189		
March [a]	0.054 ± 0.214			0.287 ± 0.200		
April [a]	-0.090 ± 0.227			0.511 ± 0.211		
May [a]	0.391 ± 0.290			0.832 ± 0.288		
June [a]	0.168 ± 0.264			0.925 ± 0.256		
July [a]	0.116 ± 0.362	**<0.001**	***	0.801 ± 0.328	**<0.001**	***
August [a]	0.051 ± 0.195			0.256 ± 0.173		
September [a]	-0.026 ± 0.185			0.447 ± 0.168		
October [a]	0.038 ± 0.217			0.300 ± 0.200		
November [a]	0.042 ± 0.242			0.171 ± 0.222		
December [a]	-0.125 ± 0.839			1.137 ± 0.819		
Pregnant (late) [b]	**0.266 ± 0.116**	**0.021**	*	-0.002 ± 0.115	0.949	
Lactating (♀ calf) [c]	-0.122 ± 0.089	0.439		0.015 ± 0.077	**0.022**	*
Lactating (♂ calf) [c]	-0.032 ± 0.090			0.229 ± 0.081		
Assay date	***8 factor levels***	**<0.001**	***	***8 factor levels***	**<0.001**	***
Time diff	**0.001 ± <0.001**	**0.007**	**	<0.001 ± <0.001	0.370	
Age:Pregnant (late) [b]	-	-		**0.057 ± 0.022**	**0.009**	**

[a] monthly variance estimates is relative to January estimates

[b] pregnancy status relative to non- & early- pregnant females

[c] lactation status relative to non-lactating females

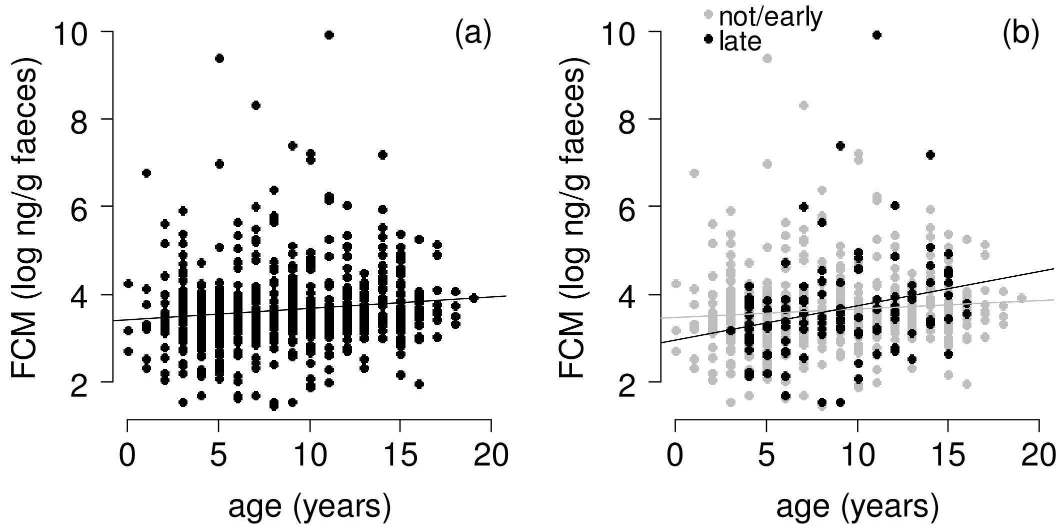


Figure 6.1: *Variation in log transformed FCM concentrations with age (a) for all the data, and (b) amongst females which were not pregnant or sampled during early pregnancy (grey) and those sampled in late pregnancy (black). The figures show the raw data with fitted regression lines from regressions of log-transformed FCM against age for (a) all the data and (b) data which was subset by pregnancy status.*

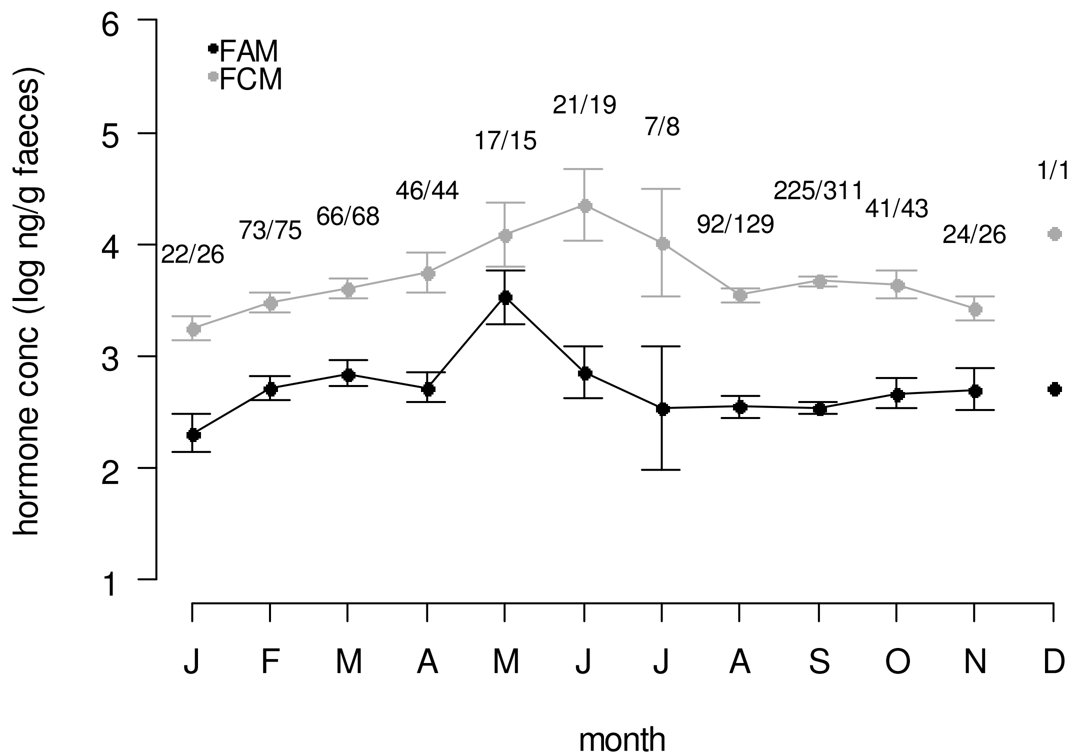


Figure 6.2*: Variation in log-transformed FAM (black) and FCM (grey) concentrations with month. Points represent monthly averages ± SE. Numbers represent monthly sample sizes for FAM/ FCM respectively. Only one sample was collected in December and so no estimate of error could be provided.*

6.5.3. Reproductive status

In addition to age- and season-related effects, this study also explored the relationships between female reproductive status and faecal hormone concentrations. Both hormones varied with pregnancy status (Table 6.1). Levels of FAM were higher during late pregnancy compared to females who were in early pregnancy or not pregnant at all (p=0.021; Table 6.1a). Late stage pregnancy was also associated with higher FCM levels but only in older age groups (p=0.009; Table 6.1b; Figure 6.1). In addition, FCM concentrations also varied depending on the female's lactation status at the time of sampling (p=0.022; Table 6.1b). Females had higher FCM when they were suckling a son than when they were suckling a daughter (which didn't appear to differ from females which were not lactating at all).

6.5.4. (Co)variance components

After accounting for the effects of age, season and reproductive state there was no evidence of either among-individual or among-year variation for either hormone (Table 6.2). There was significant within-individual variance for both hormones, however the within-individual covariance between FAM and FCM was non-significant for either early (LRT: p=0.376; Table 6.2) or late (LRT: p=0.378; Table 6.2) models. There were no repeat samples for calf testosterone so among-individual variance could not be estimated, there were also no significant within-individual covariances between calf testosterone and either maternal FAM (LRT: p=0.369-0.498; Table 6.2c) or FCM (LRT: p=0.177-0.438; Table 6.2c).

***Table 6.2**: Multivariate mixed effects model estimating variances (diagonal), covariances (below diagonal), and correlations (above diagonal) for FAM, FCM and, when females were sampled during pregnancy, circulating testosterone levels in the resulting calf at (a) among-individual, (b) among-year, and (c) residual within-individual levels (SE in parentheses). Where females were sampled more than once during pregnancy with the same calf, the last sample collected during (1) early and (2) late pregnancy (≤/> 118 days into gestation) was associated with calf testosterone levels in the within-individual covariance/correlation analyses. Shaded cells indicate values that were not estimated. Statistically significant ($p<0.05$) variances and covariances are in bold. See Table 6.1 for the fixed effects from this model.*

(1) EARLY

(a) Among-individual

	FAM	FCM	Calf T
FAM	0 (NA) X^2=0 *p=1*		
FCM		0.002 (0.013) X^2=0.012 *p=0.875*	
Calf T			

(b) Among-year

	FAM	FCM	Calf T
FAM	0.002 (0.003) X^2=1.321 *p=0.104*		
FCM		<0.001 (0.001) X^2=0.004 *p=0.927*	
Calf T			0.007 (0.016) X^2=0.631 *p=0.261*

(c) Within-individual

	FAM	FCM	Calf T
FAM	**0.663** **(0.038)** **X^2=34.693** ***p<0.001***	0.038 (0.042)	0.083 (0.154)
FCM	0.025 (0.027) X^2=0.391 *p=0.376*	**0.639** **(0.036)** **X^2=31.500** ***p<0.001***	-0.112 (0.136)
Calf T	0.031 (0.058) X^2=0.229 *p=0.498*	-0.041 (0.050) X^2=0.911 *p=0.177*	**0.210** **(0.039)** **X^2=22.923** ***p<0.001***

(2) LATE

(a) Among-individual

	FAM	FCM	Calf T
FAM	0 (NA) X^2=0 *p=1*		
FCM		0.003 (0.013) X^2=0.005 *p=0.918*	
Calf T			

(b) Among-year

	FAM	FCM	Calf T
FAM	0.002 (0.003) X^2=1.202 *p=0.121*		
FCM		<0.001 (0.001) X^2=0.006 *p=0.912*	
Calf T			0.021 (0.036) X^2=1.581 *p=0.075*

(c) Within-individual

	FAM	FCM	Calf T
FAM	**0.663** **(0.038)** **X^2=34.571** ***p<0.001***	0.037 (0.042)	-0.175 (0.145)
FCM	0.024 (0.027) X^2=0.388 *p=0.378*	**0.638** **(0.036)** **X^2=31.567** ***p<0.001***	-0.170 (0.129)
Calf T	-0.066 (0.055) X^2=0.415 *p=0.362*	-0.063 (0.049) X^2=0.300 *p=0.438*	**0.214** **(0.038)** **X^2=24.297** ***p<0.001***

6.6. Discussion

These analyses show considerable individual-level variation in female FAM and FCM levels. As with chapter 5, this variation was affected by sampling season in both hormone groups, although only FCM levels varied significantly with age. The importance of understanding and accounting for a female's reproductive status is also highlighted, as both FAM and FCM levels were influenced by the female's pregnancy status at the time of sampling, with additional effects of offspring sex on FCM levels during lactation. After accounting for the effects of age, season and reproductive status, neither hormone group showed any among-individual repeatability, neither were there any among-year trends. This suggests that variation in hormone concentration between individuals is likely to be predominantly determined by differences in their present state and environment. There was also no relationship between maternal hormone levels when pregnant and the circulating neonatal testosterone levels in the resulting calf, supporting evidence from chapters 3 & 4 which suggest that neonatal testosterone levels are more influenced by the calf than the mother.

6.6.1. Androgens

There was no effect of age on FAM levels at either the individual or population levels. This means that there was no consistent within-individual change in FAM with age, neither was there any evidence of particular FAM level phenotypes being selectively lost from the population in older age classes. FAM levels did, however, show substantial seasonal variation, peaking in May to coincide with the start of the calving season. This fits with a more general trend towards higher FAM concentrations during late gestation in these females (Table 6.1). Circulating testosterone levels have been shown to increase towards the end of gestation across a range of different species (Demisch et al. 1968, Barkley et al. 1979, Bammann et al. 1980, Gaiani et al. 1984, Silberzahn et al. 1984), which is attributed to a combination of increased steroid secretion (e.g. late gestation humans: Bammann et al. 1980, horses: Silberzahn et al. 1984), and slower hormone clearance (Bammann et al. 1980). This suggests that the seasonal variation in FAM concentrations is more likely to be a consequence of physiological changes during pregnancy, rather than extrinsic

environmental cycles. The absence of any lactation effect on FAM concentrations indicates that in these females, the physiological changes associated with lactation are not reflected in circulating androgen levels. This may, in part, be a consequence of suckling-induced prolactin secretion, which has been shown to reduce ovarian activity in humans (Alder et al. 1986) and thus may rapidly return FAM to pre-pregnancy levels.

Despite evidence for short-term among-individual repeatability of testosterone levels in other species (Pelletier et al. 2003, While et al. 2010), there was no evidence of repeatability of FAM levels amongst individual females in this study. This is supported by both previous results from male red deer in this population (chapter 5), and with evidence from other studies showing endocrine measures to become less repeatable over longer time periods (e.g. Boulton et al. 2015). The results presented in this chapter may therefore differ from other studies because samples were collected over several years, whilst previous studies have been more interested in repeatability within shorter time periods of days (e.g. Pelletier et al. 2003) or months (e.g. While et al. 2010). The lack of temporal autocorrelation (Table F. 4 & Table F. 5) suggests that there was no trend in the correlation over time (i.e. the correlation between samples did not decrease as time increased). This, coupled with the fact that samples collected 3 days apart had a correlation of <0.01 (Table F. 5), suggests that the lack of repeatability in this data is not a result of large time differences between some samples from the same individual.

6.6.2. Glucocorticoids

In contrast to FAM, there were clear age-related trends in FCM concentrations. The within-individual increase in FCM levels with age is consistent with the aging effect seen in FCM levels within males in chapter 5. Such within-individual change may arise due to desensitisation of the cortisol stress response, leaving cortisol concentrations elevated for longer periods after a stressor (Sapolsky et al. 1984, 1986). Whilst FCM appeared to increase within individuals as they aged, females who reached an older age of last sample had lower FCM levels on average. This suggests the selective disappearance of high FCM individuals, although the small

effect size (-0.042 ± 0.016 SE; Table 6.1) indicates that the effect is a weak one. Given that high cortisol levels are associated with reduced fitness (Romero and Butler 2007), the selective disappearance of high FCM females with age could be indicative of reduced lifespans, although the dataset was collected too recently to fully test this as most individuals are still alive. Rather than being biological in origin, it is also possible for this trend to arise from sampling bias, i.e. older females with high FCM levels are still alive but less likely to be sampled for some, as yet unknown, reason.

Whilst there was no consistent variation amongst years, there were clear seasonal cycles, with FCM peaking in May-June to coincide with the calving season. This concurs with findings from other taxa which exhibit increased glucocorticoids towards the end of gestation (McMillen et al. 1987, Atkinson and Waddell 1995, Cavigelli 1999, Obel et al. 2005). High FCM levels might either arise due to an absolute increase in circulating glucocorticoids levels or due to increased sensitivity of the acute stress response (e.g. Scott et al. 1990, Obel et al. 2005).

Whilst there is no overall variation in FCM levels with pregnancy status, older females had significantly higher levels during late gestation than non-pregnant females of a similar age (Figure 6.1b). This is likely to occur due to pregnancy-linked stressors (which arise as a consequence of the high cost of reproduction, Catchpole et al. 2004) being amplified by the age related desensitisation of the stress response. In concurrence with previous studies (Hill et al. 2003, Ryan et al. 2012), there was no overall difference in FCM levels between lactating and non-lactating females, however mothers suckling a son had much higher FCM levels than those who had a daughter, or who were not lactating at all. These high FCM levels suggest an overall increase in stress levels when rearing a son versus rearing a daughter (or not having a calf at all), possibly as a result of sons being more costly to rear than daughters (Clutton-Brock et al. 1981) because of their greater body weight (Clutton-Brock et al. 1982) and higher milk demands (Landete-Castillejos et al. 2005).

In direct contrast to male red deer (chapter 5), there was no evidence of repeatability of FCM levels amongst individual females. In fact, a model where repeatability was constrained to be the same in both sexes was significantly worse than a model when

it was allowed to differ (after standardising for sex-specific differences) (LRT: $\chi^2_{(1)}$=3.583; p=0.007; Table F. 7), demonstrating higher among-individual variance in males than females. This suggests that female FCM levels fluctuated more acutely with varying conditions than males, possibly influenced by changes in the sensitivity of the stress response at different stages of the reproductive cycle (Scott et al. 1990, Obel et al. 2005).

In concurrence with the males in chapter 5, there was no within-individual covariance between FAM and FCM levels, and the absence of among-individual and among-year variance for these hormones meant that the covariances between these couldn't be tested. This study therefore found no evidence for a relationship between a female's FAM and FCM levels: females with high concentrations of one hormone were no more likely to have higher concentrations of the other. This suggests that, contrary to some studies which show stress (and associated high glucocorticoids) to inhibit testosterone secretion (e.g. Wingfield and Silverin 1986, Perez-Rodriguez et al. 2006), concentrations of FAM in these female red deer do not exhibit predictable changes with stress.

6.6.3. Maternal hormones & offspring testosterone

No relationship was found between a calf's testosterone levels and the mother's faecal hormone concentrations during foetal development. This concurs with chapter 3 which suggests neonatal testosterone levels to be more indicative of the state of the calf rather than the state of the mother. It does, however, rely on the assumption that neonatal testosterone levels were representative of levels during foetal development, which may not be the case. This study, along with results from chapter 5 (Table 5.2), showed no evidence of an individual's FAM levels being repeatable across their lifetime, although this has not been tested for circulating hormones in foetal and early life.

The absence of a correlation between maternal FAM and calf testosterone levels could also be used to support previous findings in rodents and cattle (Slob et al. 1980, Vreeburg et al. 1981, Tapanainen 1983) which suggest that neonatal

testosterone is endogenously produced, rather than being a consequence of mother-to-foetus transfer. This assumes that FAM concentrations reflect circulating testosterone levels in the female. Whilst this is often the case (Bishop and Hall 1991), there are a number of problems with this assumption as these two methods are fundamentally measuring different hormones over different timescales (see chapter 1.4 for detailed discussion).

6.6.4. Sampling effects

Given the high bacterial load in faeces, faecal steroids are generally considered less stable than those in blood or urine samples (Whitten et al. 1998). If not accounted for, this can introduce considerable noise and error into measures of faecal hormone concentration. The increase in FAM levels with time to freezing suggests that faecal bacteria were synthesising androgens from other faecal steroids. Although the effect size was small, the statistical significance indicates that concentrations of the androgens measured in this assay (i.e. captured by the antibody) and their precursors cannot be considered stable, even within hours of defecation, and should be frozen at -20ºC as soon as possible. By contrast, FCM concentrations remained more stable across time, indicating that the cortisol metabolites were less prone to the effects of bacterial synthesis during the first day of faecal collection. There was, however, a trend towards higher FCM levels with increased time to freezing, indicating the care should still be taken to minimise time between defecation and freezing. In concurrence with previous studies, assay date also explained significant variation between samples (Graham et al. 2010; see also chapter 3). This could be a consequence of variation in laboratory conditions (e.g. lab temperature) between days (Graham et al. 2010, Watson et al. 2013).

6.6.5. Conclusion

In summary, both androgen and cortisol metabolite concentrations varied considerably between samples. Only FCM levels showed variation with a female's age, but both FAM and FCM showed pronounced seasonal cycles, with concentrations peaking around the spring calving season. Reproductive status was also important in explaining variation between samples. Both hormone groups were

higher during late pregnancy, although FCM levels only showed this trend in older females where physiological aging of the cortisol response may amplify pregnancy-linked stressors. Lactating FCM concentrations were also higher when mothers suckled a male calf, possibly due to the higher energetic cost of raising a son. After accounting for the effects of age, season and reproductive status, neither hormone group showed any among-individual or among-year variance, and neither influenced calf testosterone levels when measured in their pregnant mothers. This indicates that individual differences in current state and environment are most important in determining measured hormone levels, and that neonatal calf testosterone levels are more likely a reflection of calf, rather than maternal hormone levels.

7 | Summary

Although the importance of understanding individual hormone variation is increasingly being acknowledged, many studies continue to overlook it or to treat it as "noise" around a population mean (see Williams 2008 for discussion). In this thesis, I utilised both traditional (blood) and non-invasive (faecal) sampling methods to show how levels of two steroid hormone groups, androgens and glucocorticoids, differed substantially between individual wild red deer. The degree to which individuals varied from one another illustrates the value of considering variation at the individual level, as well as how life history, ecology and local environment combine to shape an animal's hormone responses. My results further showed that deer not only vary considerably from one another, but also vary temporally as an individual's external and internal conditions changed. These individual differences in state and responses need to be accounted for before fitness implications of differences in hormone levels can be explored.

In this concluding chapter, I draw together the results from chapters 3-6 and discuss how they contribute to our broader understanding of the causes and effects of hormone variation. These previous chapters were organised by hormone type: first circulating testosterone levels (chapters 3 & 4) and then levels of faecal androgen and cortisol metabolites (FAM & FCM in chapters 5 & 6). Now, however, I draw from across these chapters to address extrinsic (environment & age), intrinsic, fitness-linked, and methodological effects in turn. The results are first summarised in Table 7.1, and then discussed in detail in the following sections.

***Table 7.1:** Causes and effects of variation in (a) circulating neonatal testosterone (chapters 3 & 4) and (b) faecal androgen and cortisol metabolites (chapters 5 & 6). Significant effects are denoted by "✓" and non-significant effects by "×". Untested traits are marked "NA".*

	(a) Blood plasma Testosterone			**(b) Faecal**			
				FAM		**FCM**	
Sex	Both	Male	Female	Male	Female	Male	Female
Age range	0-14 d.o.	0-14 d.o.	0-14 d.o.	1-14 y.o.	0-19 y.o.	1-14 y.o.	0-19 y.o.
Environmental effects							
Year	✓	✓	✓	×	×	×	×
Month	NA	NA	NA	✓	✓	✓	✓
Time of collection	✓	✓	×	×	×	×	×
Age							
Age	✓	✓	✓	NA	×	✓	✓
Age^2	×	×	×	✓	×	×	×
Intrinsic state							
Sex	✓	NA	NA	NA	NA	NA	NA
Genetic variation	✓	✓	✓	×	×	×	×
Pregnancy status	NA	NA	NA	NA	✓	NA	✓
Lactation status	NA	NA	NA	NA	×	NA	✓
Maternal identity	×	×	×	NA	NA	NA	NA
Maternal age	×	×	×	NA	NA	NA	NA
MRS	×	×	×	NA	NA	NA	NA
Sex of prev. sibling	✓	✓	×	NA	NA	NA	NA
Fitness-linked traits							
Calf survival	✓	✓	×	NA	NA	NA	NA
Reproductive effort	NA	NA	NA	×	NA	✓	NA
Methodology							
Assay date	✓	✓	✓	✓	✓	✓	✓
Time to freezing	NA	NA	NA	×	✓	×	×

d.o.: days old
y.o.: years old

7.1. Environmental effects

Throughout this thesis, hormone levels in the Rum red deer have been shown to exhibit pronounced temporal variation at multiple nested scales. The following sections outline the hormone variation observed at annual, seasonal and daily levels within this population, and discuss it in light of potential environmental drivers.

7.1.1. Annual variation

Chapters 3 & 4 showed circulating testosterone levels to vary considerably between neonatal calves born in different years. Such cohort variation may arise as a

consequence of climatic variation or maternal food availability during foetal development or very early life. This is supported by preliminary analyses in appendix B, which identified key time windows in foetal development when weather conditions significantly influenced the neonatal testosterone levels of the resulting calf (see appendix B for further discussion of potential weather effects). In contrast, however, after accounting for age and seasonal effects, faecal metabolite levels showed no clear annual variation (chapters 5 & 6). This suggests that when scaling up to consider the population across the entire year, recent conditions and intrinsic state were more important in explaining faecal metabolite concentrations than differences in conditions between years.

7.1.2. Seasonal/ circannual variation

Where deer were sampled across the year (chapters 5 & 6), rather than just within a particular season (as in chapters 3 & 4), a clear circannual rhythm was detected in both androgen and glucocorticoids levels (Figure 5.1 & Figure 6.2). These cycles are likely driven by a combination of stimuli, including weather, photoperiod, food availability and stage in the reproductive cycle (see chapters 5.6 & 6.6 for sex-specific discussions).

My results suggest that the reproductive cycle was the most important influence on the seasonal variation observed in levels of both androgens and glucocorticoids. Female FAM and FCM peaked during the spring calving season (Figure 6.2), whilst males exhibited the highest levels of both hormones during the autumn rut (Figure 5.1). The increase in female FAM levels towards parturition in this population (Table 6.1a) agreed with changes seen in other ungulate species (e.g. Gaiani et al. 1984, Silberzahn et al. 1984), which have previously been attributed to greater secretion (Silberzahn et al. 1984) and slower clearance (Bammann et al. 1980) of hormones during late gestation. The peak in female FCM levels during the calving season is more likely a reflection of the stress and physiological demands of a developed foetus and its subsequent birth (e.g. McMillen et al. 1987, Atkinson and Waddell 1995, Cavigelli 1999, Obel et al. 2005; see also chapter 6.6.2 for discussion). In stags, the rutting season is characterised by intense male-male competition for

sexually receptive females (Clutton-Brock et al. 1979). The association between heightened aggression and increased levels of both androgens (Clutton-Brock et al. 1979) and glucocorticoids (e.g. Muller and Wrangham 2004b) in other studies indicates that the circannual cycle I observed in the stags (see Figure 5.1) is most likely linked to changes to their social environment and behaviour during the rutting period (see chapter 5.6.2 for discussion).

After considering associations with the reproductive cycle, stags also exhibited a second, smaller peak in FCM levels during March when the Rum population begins emerging from winter (Figure 5.1). This also coincides with the start of antler regrowth in the Rum stags (Figure 1.4), which is expected to be very energetically costly (Clutton-Brock et al. 1979, Lemaitre et al. 2014). Winter is a particularly tough time for the deer on the Isle of Rum (Clutton-Brock et al. 1982), but the fact that only the males exhibited an increase in FCM levels during this time period suggests that they are under more physiological stress during this time than females. Males are larger and require more resources, both because of size-related metabolic demands (Clutton-Brock et al. 1982), and the demands of antler growth (Clutton-Brock et al. 1979). It therefore stands to reason that males would be more vulnerable to harsh winter conditions than females.

7.1.3. Daily/ circadian variation

Concurrent with other red deer studies (Lincoln and Kay 1979, Asher and Peterson 1991, Ingram et al. 1999), I found clear circadian hormone rhythms in the wild Rum red deer, although daily variation was only detectable in circulating hormones. This is unsurprising given that hormone metabolites in faecal samples provide an aggregate measure across several hours and so would not detect such acute hourly changes over time (see chapter 1.4 for discussion). As a consequence, my analyses only found circadian rhythms in neonatal testosterone levels (discussed in chapter 3.6.3), as blood samples are not collected from adults in this wild population.

7.2. Within-individual aging

After correcting for environmental effects, temporal variation in hormone concentration may also arise as a consequence of within-individual changes over an animal's lifetime.

7.2.1. Androgens

In this study system, calves of both sexes were born with relatively high levels of circulating testosterone, which rapidly declined over the first 24 hours of life (Figure 3.1), possibly as a consequence of slower clearance rates in the hours following birth (Baum et al. 1988). Longer term trends could also be observed in male FAM levels, which increased with age until stags were around 7-10 years old, after which FAM levels declined (Figure 5.2). This coincides with prime reproductive age (~8-11 years old: Nussey et al. 2009; see also Figure 5.3) when stags are more likely to interact with sexually receptive females and to engage in aggressive behaviours, both of which have previously been shown to positively correlate with circulating testosterone levels (Lincoln et al. 1972, Macrides et al. 1974, Book et al. 2001, Muller and Wrangham 2004a). By this argument, we would expect associations between measures of annual reproductive effort and FAM levels. Such a scenario could not be formally tested, however, because of the lack of among-male FAM repeatability (chapter 5).

There was no evidence of any age-related trends in FAM levels within females, however, where reproductive success is not so strongly age dependent (chapter 6). Whilst some factors associated with age might influence a female's fertility (Albon et al. 1986), all females have the opportunity to breed annually from around 3 years old (Clutton-Brock et al. 1982). Other factors such as a female's reproductive history also play a large role in determining their annual reproductive success (Albon et al. 1986).

7.2.2. Glucocorticoids

Both sexes showed the same within-individual increase in FCM levels with age (Figure 5.2b & Figure 6.1). This is consistent with previous studies which show

circulating glucocorticoid levels to increase in rats (Sapolsky 1991) and humans (Halbreich et al. 1984, van Cauter et al. 1996) over time, presumably due to the physiological aging of the stress response. As animals age, the hypothalamus becomes less sensitive to the stress feedback loop which lowers circulating glucocorticoid levels back to baseline after a stressor (Sapolsky et al. 1984, 1986). Overall, circulating levels in older animals therefore remain higher for longer.

7.3. Effect of intrinsic state

In addition to the temporal variation discussed in the previous sections (chapter 7.1 & 7.2), hormone concentrations also varied due to more persistent differences between individuals. Here I discuss how androgen and cortisol concentrations varied due to sex, genetic variation, current reproductive state, and non-genetic maternal effects.

7.3.1. Sex differences

In polygynous species, adult males almost always have considerably higher circulating testosterone levels than adult females (Kempenaers et al. 2008). This may arise, in part, from the positive relationship between testosterone levels and traits such as aggression and dominance which are known to be important for mate acquisition (Oliveira 2004, Kempenaers et al. 2008). Whilst this sex difference was present in FAM levels measured across all age groups (t-test between uncorrected, log-transformed data: $t=-10.544$, $df=162$, $p<0.001$), male *calves* also had higher circulating testosterone levels than females of the same age (Table 3.1; chapter 3). Often referred to as a "male sex hormone" because of its strong association with male reproductive traits, testosterone is rarely studied in juvenile individuals (though see Civantos 2002, Cox et al. 2005). Analyses here, however, showed substantial trait-linked variation even in early life levels (chapter 3). Despite calves averaging only 1.9 days old when first sampled, males already had higher circulating concentrations of testosterone than females ($p=0.002$; Table 3.1), although levels appeared to be highly genetically correlated across the sexes (chapter 4). Whilst this sex difference is conserved across all age groups and sample types, it is important to note that sex differences in faecal androgen metabolite levels might

also be influenced by differences in the relative proportions of metabolites produced (as discussed in reviews by Palme 2005, Goymann 2012).

In contrast to testosterone, cortisol showed less consistent sex differences. There was no statistical difference between circulating cortisol levels in the subset of male and female calves sampled (appendix A). Despite females having a higher mean FCM level (female mean: 106.4 ng/g faeces ± 31.9 SE; male mean: 61.5 ng/g faeces ± 6.3 SE), there was also no statistical sex difference in uncorrected, log-transformed FCM concentrations (t-test between uncorrected, log-transformed data: $t=-0.958$, $df=270$, $p=0.339$). As with FAM levels, however, caution has to be taken in drawing direct comparisons between faecal metabolite levels because it is not known whether the metabolites measured by the EIA antibodies are produced in the same ratios in both sexes.

7.3.2. Genetic variation

The results presented here showed that genetics also played a role in determining an individual's eventual circulating hormone levels, at least in very early life. Chapter 4 showed 16% of individual variation in circulating testosterone levels to be genetically heritable in calves. This did not appear to be the case across the board however, as FAM levels were not repeatable amongst individuals (chapters 5 & 6) and therefore exhibited no consistent (or heritable) differences (appendix C). FCM levels were also not repeatable among females, and whilst individual differences were observed in male FCM levels, these too showed no evidence of genetic heritability (appendix C). Concentrations of faecal hormone metabolites are arguably less likely to be heritable because they are not only determined by hormone secretion (as with circulating hormone levels), but also by diet, metabolism and gut biota (see Table 1.2 and chapter 1.4 for discussion). These may all vary both within and between individuals (Goymann 2012), introducing a suite of potentially independent traits which result in the eventual measured concentrations.

7.3.3. Reproductive state

In females some of the greatest physiological changes occur during pregnancy and subsequent lactation, with reproductive state closely associated with a female's hormone levels (see chapter 6). Both FAM and FCM levels increased in late pregnancy in females, although this only affected FCM levels in older females (Figure 6.1b) where pregnancy-linked stressors may be amplified by the physiological aging of the stress response (see chapter 6.6.2 for discussion). Lactating FCM levels were also higher when females suckled a male rather than a female calf (Table 6.1), possibly due to the higher energetic costs of raising a son (Clutton-Brock et al. 1981). These results show the importance of understanding a female's current reproductive state when analysing her hormone levels, as well as supporting previous evidence of sex differences in the cost of suckling calves in this species (Clutton-Brock et al. 1981, Clutton-Brock et al. 1982, Landete-Castillejos et al. 2004).

7.3.4. Maternal effects

Maternal effects on offspring hormone levels (over and above any effects of inherited genes) might arise as a consequence of direct hormone transfer (as is seen in birds: e.g. Schwabl 1993, Groothuis and Schwabl 2008), due to an intrinsic property of the mother, or as a consequence of a transient maternal state (discussed in chapter 1.3.2). There was, however, little evidence in this study to suggest that maternal effects had any major direct influence on the circulating hormone levels of offspring via any of these means (chapter 3). This indicates that even from an early age, an individual's hormone concentrations were more indicative of their own intrinsic state than that of their mother.

There was also no evidence of maternal identity explaining any variance seen between circulating neonatal testosterone levels: calves were no more similar to maternal siblings than to any other individual sampled. This indicates that a mother's intrinsic state had no substantial influence on her offspring's hormone levels. There is also little evidence in the literature to suggest that androgens are passed from mother to offspring during foetal development, with the placenta

appearing to act as a barrier to a number of hormones, including testosterone (Vreeburg et al. 1981, Slob et al. 1983, Sathishkumar et al. 2011, Sun et al. 2012). This would prevent the deposition of maternal hormones into the circulatory system of the developing foetus. I therefore concluded in chapter 3 that the measured concentrations of circulating neonatal testosterone were the result of calf secretions, and thus reflected calf (not maternal) levels (see chapter 3.6 for discussion). This conclusion is supported by the absence of any correlation between a female's FAM levels whilst pregnant and the circulating testosterone levels in the resulting calf (chapter 6), even after correcting for gestation time.

The only maternal trait found to significantly influence offspring hormone levels was the sex of the previous calf carried by the mother (Table 3.1). Circulating testosterone levels were lower in calves preceded by an older brother compared to those which were firstborns or born after a sister. In red deer, male calves are more energetically expensive (see chapters 3.6.2 & 6.6.3 for discussion), so this could be a consequence of depleted maternal resources meaning that mothers had less to invest in the subsequent calf. If this were purely a reflection of maternal quality or condition, however, we would expect other traits such as reproductive status and maternal age to also have an effect, as these are known to influence maternal condition (Clutton-Brock et al. 1982, 1983, Albon et al. 1986, Nussey et al. 2006).

7.4. Fitness-linked trait expression

Numerous experimental studies show experimentally elevated levels of both androgens and glucocorticoids to effect components of fitness when compared to control groups (discussed in chapter 1.2.1). These studies, however, usually just focus on comparing one experimentally manipulated (e.g. hormone implanted) treatment group with a control group. Whilst this approach allows questions about causation to be asked, it does not take into consideration the individual variation occurring within each treatment group. In my thesis I ask whether the hormone-fitness effects observed in other systems with large-scale manipulation, can also be seen at the level of the individual through finer-scale naturally-occurring levels of variation. Overall this did not appear to be the case. Whilst there were some links

found between hormone concentrations and fitness-linked traits, there was little evidence of wide-scale hormone-linked fitness effects in this wild population. Below, I discuss the results of analyses exploring the possible relationships between a deer's androgen or cortisol levels and metrics of survival and reproductive effort.

7.4.1. Survival

Juvenile survival (survival to one year old) was one of the few fitness-linked effects of hormone concentration found in this thesis, (chapter 3). Firstborn calves, particularly males, with higher than average concentrations of circulating testosterone were more likely to die before reaching one year old than were firstborns with lower than average testosterone levels (Figure 3.2). This concurs with findings from experimental studies in adults, where high testosterone levels are associated with negative effects such as immunosuppression, elevated parasite load, and reduced survival in males of various taxa (Owen-Ashley et al. 2004, Reed et al. 2006, Decristophoris et al. 2007, Malo et al. 2009).

I could not directly test the relationship between hormones and survival in adults because only a few sampled individuals had died naturally before the end of the study, and any analyses would risk being biased towards individuals dying young. It was, however, possible to test for population-level aging patterns by investigating the selective disappearance of particular hormone phenotypes from the sampled population. The disappearance of individuals with particular hormone phenotypes (e.g. relatively high or relatively low for the population) might be indicative of the selective mortality of that phenotype (van de Pol and Verhulst 2006). After correcting for the within-individual FCM aging patterns (discussed in chapter 7.2.2), population-level FCM concentrations were found to decline with age in females (Table 6.1). This suggests that females with high FCM levels were lost from the sampled population at a younger age. Higher mortality rates in individuals with high cortisol levels concurs with previous studies where glucocorticoids have been positively linked to reduced immune function (Zysling et al. 2006)(Zysling et al. 2006)(Zysling et al. 2006)(Zysling et al. 2006)(Zysling et al. 2006) and cellular damage (Sapolsky et al. 1986). A population-level decrease in mean FCM levels with

age could either be the result of cortisol-linked mortality, or an artefact of sampling. The introduction of sampling bias seems unlikely given the combination of *ad hoc* faecal collection and the targeting of particular individuals. The lack of among-individual repeatability in female FCM levels (chapter 6), however, also indicates the absence of any fixed FCM female phenotypes, leaving this issue unresolved.

7.4.2. Reproductive effort

Based on previous studies (Lank et al. 1999, Peters et al. 2001, Reed et al. 2006), variation in testosterone levels was expected to account for some variation in reproductive performance. This is particularly true for males, where high concentrations of testosterone have been linked to increased reproductive traits in a number of different species (see chapter 5 for discussion). No such relationships were found here, however. FAM levels were not repeatable in either males or females (Table 5.2 & Table 6.2), so no fixed FAM phenotypes could be assigned. Instead it appears that FAM levels were more dependent on the recent/current state or environment of the individual. As a result, they were not consistent enough to influence annual reproductive traits, let alone lifetime measures.

Whilst a stag's FCM levels did positively correlate with their annual reproductive effort (Figure 5.4) this is unlikely to be a causal relationship, but rather one driven by the effects of social dominance. Dominant stags would invest the most effort during the reproductive season, engaging in male-male conflict in defence of their harem (Lincoln et al. 1972, Clutton-Brock et al. 1982). Given that stags retain dominance ranks outside of the breeding season too (Clutton-Brock et al. 1982), these dominant individuals would also likely engage in the most male-male conflict across the year, leading to elevated cortisol levels as a result (see chapter 5.6 for discussion).

7.4.3. Scope for sexually antagonistic selection

Optimal testosterone concentrations are expected to differ between the sexes due to the sex-specific effects of testosterone on numerous fitness-linked traits (discussed in chapters 1.2.2 & 4.2.2 amongst others). If individual testosterone levels are both

heritable and caused by the same genes in both sexes, then there is the potential for sexually antagonistic selection to occur because the sexes are constrained from evolving independently. The implications of this for testosterone levels have been widely discussed in the literature, although the focus has almost entirely been on adult testosterone levels (e.g. in birds: Moller et al. 2005, fish: Mank 2007, and mammals: Mills et al. 2012). In chapter 4 I showed circulating neonatal testosterone levels to be heritable in the Rum red deer calves, and to be genetically correlated between the sexes. Whilst this provides the genetic basis for sexually antagonistic selection on neonatal testosterone, there is no evidence to suggest that there are the selection pressures or between-sex conflict necessary to drive this. The effects of neonatal testosterone on survival were only found for a subset of calves, and therefore selection on this trait is likely to be very weak. Furthermore, there was no evidence of divergent sexual optima for neonatal testosterone levels (chapter 3). Due to the lack of any heritable genetic variance in either FAM or FCM levels (appendix C), there is no scope for selection to act on either of these traits.

7.5. Methodological artefacts

This thesis also explored potential methodological errors which may have confounded biological results. The largest effects were not found in the handling and processing of the biological samples, but when assaying hormone extracts in the laboratory. All analyses found the date of assay to account for a highly significant level of variation between samples (chapter 3.5, 5.5 & 6.5). Despite the apparent importance of correcting for assay date in these analyses, the potential effects of laboratory conditions on measured hormone concentrations are seldom acknowledged in publication (though see Watson et al. 2013). It is unknown whether this is because it is overlooked as a potential cause of variation, or tested and excluded due to non-significance. Given that I ran multiple assay plates on any given day, the between-day effects could be separated from between-plate effects (measured as inter-assay CV) which are more likely to reflect error introduced by the researcher. In all analyses presented here, the mean within-day inter-assay CVs were consistently lower than the overall inter-assay CV (based on plate controls) (chapter 2). This further supports evidence from the models that the between-day

differences were not just reflecting error introduced between plates. The repeatability of assay reactivity between days has previously been explored in antibodies, where differences have been attributed to variation in laboratory temperature when loading plates on different days (Graham et al. 2010).

The one sampling/storage artefact identified in this study was the increase in measured androgen metabolite levels in faecal samples kept at ambient temperature for longer. Metabolite concentrations may increase in faecal samples over time due to microbial breakdown of precursor hormones in the faeces (Mostl et al. 1999). Evidence from this study suggests that faecal samples should be frozen within 4 hours of collection to reduce the risk of storage effects on FAM levels, however details of time at ambient should always be recorded to test for this. It should be noted that this trend was only small (Est: 0.001 ± <0.001) and only significant in the female models, although this might be an artefact of sample size (638 female samples versus 141 male samples).

7.6. Conclusion

In this thesis, I have shown the considerable degree to which individuals differ in their hormone concentrations, and the importance of being able to account for specific causes of that variation when exploring fitness-linked consequences. An individual's hormone levels at a given point in time are due to a combination of both their local extrinsic and intrinsic environments. The potential confounding effects of variation in an individual's local environment and recent experiences, therefore, need to be considered and understood before drawing more general conclusions.

Temporal variation appears to exert a strong influence over an individual's hormone levels at multiple scales. Neonatal testosterone levels varied between cohorts, and both FAM and FCM levels exhibited clear seasonal cycles, peaking during key reproductive events: the calving season in females and the rut in males. Age also had a strong effect on an individual's measurable hormone levels; with testosterone levels declining rapidly within the first day of life, FCM levels

increasing with age, and FAM peaking in middle-aged stags. An individual's current life history state also appeared to be important, particularly a female's reproductive state. Whilst there was some evidence of maternal effects on neonatal hormone levels, these were minimal, and maternal hormone concentrations did not appear to influence those in their new-born calves.

There were also associations between an individual's hormone levels and components of fitness, although these were only apparent amongst short-term fitness measures or proxies such as reproductive behaviour. Effects were not, however, ubiquitous within the population, and the limited evidence of persistent hormone phenotypes (indicated by the lack of among-individual variance for most measures) emphasises the importance of repeatedly sampling individuals. Whilst a calf's neonatal testosterone levels indicated their probability of surviving their first year of life, these effects were only apparent in firstborn males, a group which is particularly vulnerable to mortality. This suggests that the fitness consequences identified by broad-scale hormone manipulation studies can still be found when looking at individual-level differences, though effects may be subtle and not ubiquitous. The analyses presented in this thesis ultimately show that hormone levels can vary hugely between individuals, and far from just being error around a population mean, this variation is incredibly valuable in better understanding a wild population's ecology.

8 | References

Albon, S., T. Clutton-Brock, and F. Guinness. 1987. Early development and population dynamics in red deer. II. Density-independent effects and cohort variation. Journal of Animal Ecology **56**:69-81.

Albon, S., B. Mitchell, B. Huby, and D. Brown. 1986. Fertility in female red deer (Cervus elaphus): the effects of body composition, age and reproductive status. Journal of Zoology **209**:447-460.

Alder, E., A. Cook, D. Davidson, C. West, and J. Bancroft. 1986. Hormones, mood and sexuality in lactating women. British Journal of Psychiatry **148**:74-79.

Altemus, M., P. Deuster, E. Galliven, C. Carter, and P. Gold. 1995. Suppression of hypothalmic-pituitary-adrenal axis responses to stress in lactating women. Journal of Clinical Endocrinology and Metabolism **80**:2954-2959.

Appleby, M. 1980. Social dominance and food access in red deer stags. Behavior **74**:294-309.

Appleby, M. 1982. The consequences and causes of high social rank in red deer stags. Behavior **80**:259-273.

Armario, A., and J. Castellanos. 1984. Effect of acute and chronic stress on testosterone secretion in male rats. Journal of Endocrinological Investigation **7**:659-661.

Asher, G., and A. Peterson. 1991. Patterns of LH and testosterone secretion of adult male fallow deer (Dama dama) during the transition into the breeding season. Journal of Reproduction and Fertility **91**:649-654.

Atkinson, H., and B. Waddell. 1995. The hypothalamic-pituitary-adrenal axis in rat pregnancy and lactation: circadian variation and interrelationship of plasma adrenocorticotropin and corticosterone. Endocrinology **136**:512-520.

Bammann, B., C. Coulam, and N. Jiang. 1980. Total and free testosterone during pregnancy. American Journal of Obstetrics and Gynecology **137**:293-298.

Barkley, M., I. Geschwind, and G. Bradford. 1979. The gestational pattern of estradiol, testosterone and progesterone secretion in selected strains of mice. Biology of Reproduction **20**:733-738.

Barrell, G., L. Thrun, M. Brown, C. Viguie, and F. Karsch. 2000. Importance of photoperiodic signal quality to entrainment of the circannual reproductive rhythm of the ewe. Biology of Reproduction **63**:769-774.

Bartos, L., D. Schams, G. Bubenik, R. Kotrba, and M. Tomanek. 2010. Relationship between rank and plasma testosterone and cortisol in red deer males (Cervus elaphus). Physiology and Behavior **101**:628-634.

Bates, D., M. Maechler, and B. Bolker. 2011. Linear mixed-effects models using S4 classes.

Bates, R., D. Buchanan, R. Johnson, R. Wettman, R. Fent, and I. Hutchens. 1986. Genetic parameter estimates for reproductive traits of male and female littermate swine. Facultative Papers and Publications in Animal Science. **Paper 40.**

Baum, M., T. Brand, M. Ooms, T. Vreeburg, and A. Slob. 1988. Immediate postnatal rise in whole body androgen content in male rats: correlation with increased testicular content and reduced body clearance of testosterone. Biology of Reproduction **38**:980-986.

Becker, J., S. Breedlove, D. Crews, and M. McCarthy. 2002a. Introduction to behavioural endocrinology. Pages 3-38 Behavioural Endocrinology. Massachusetts Institute of Technology, Massachusetts.

Becker, J., S. Breedlove, D. Crews, and M. McCarthy. 2002b. Neuroendocrinology of sexual behavior in the female. Pages 117-152 Behavioral Endocrinology. Massachusetts Institute of Technology, Massachusetts.

Berube, C., M. Festa-Bianchet, and J. Jorgenson. 1996. Reproductive costs of sons and daughters in Rocky Mountain bighorn sheep. Behavioral Ecology **7**:60-68.

Bishop, C., and M. Hall. 1991. Non-invasive monitoring of avian reproduction by simplified faecal steroid analysis. Journal of Zoology **224**:649-668.

Bogaert, V., Y. Taes, P. Konings, K. Van Steen, D. De Bacquer, S. Goemaere, H. Zmierczak, P. Crabbe, and J. Kaufman. 2008. Heritability of blood concentrations of sex-steroids in relation to body composition in young adult male siblings. Clinical Endocrinology **69**:9-135.

Bonacic, C., D. Macdonald, and G. Villouta. 2003. Adrenocorticotrophin-induced stress response in captive vicunas (Vicugna vicugna) in the Andes of Chile. Animal Welfare **12**:369-385.

Bonier, F., P. Martin, I. Moore, and J. Wingfield. 2009a. Do baseline glucocorticoids predict fitness? Trends in Ecology and Evolution **24**:634-642.

Bonier, F., I. Moore, P. Martin, and R. Robertson. 2009b. The relationship between fitness and baseline glucocorticoids in a passerine bird. General and Comparative Endocrinology **163**:208-213.

Book, A., K. Starzyk, and V. Quinsey. 2001. The relationship between testosterone and aggression: a meta-analysis. Aggression and Violent Behavior **6**:579-599.

Boonstra, R. 2013. Reality as the leading cause of stress: rethinking the impact of chronic stress in nature. Functional Ecology **27**:11-23.

Bortolotti, G., T. Marchant, J. Blas, and S. Cabezas. 2009. Tracking stress: localisation, deposition and stability of corticosterone in feathers. The Journal of Experimental Biology **212**:1477-1482.

Boulton, J., E. Couto, A. Grimmer, R. Earley, A. Canario, A. Wilson, and C. Walling. 2015. How integrated are behavioral and endocrine stress response traits? A repeated measures approach to testing the stress-coping style model. Ecology and Evolution.

Bowman, L., S. Dilley, and E. Keverne. 1978. Suppression of oestrogen-induced LH surges by social subordination in talapoin monkeys. Nature **275**:56-58.

Brook, B., and J. Kikkawa. 1998. Examining threats faced by island birds: a population viability analysis on the capricorn silvereye using long term data. Journal of Applied Ecology **35**:491-503.

Brown, R. 1994. An Inroduction to Neuroendocrinology. Cambridge University Press, UK.

Bubenik, G., K. Miller, A. Lister, D. Osborn, L. Bartos, and G. Van der Kraak. 2005. Testosterone and estradiol concentrations in serum, velvet skin, and growing antler bone of male white-tailed deer. Journal of Experimental Zoology **303A**:186-192.

Bubenik, G., and D. Schams. 1986. Relationship of age to seasonal levels of LH, FSH, prolactin and testosterone in male white-tailed deer. Comparative Biochemistry and Physiology Part A: Physiology **83**:179-183.

Buchanan, K., M. Evans, A. Goldsmith, D. Bryant, and L. Rowe. 2001. Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling? Proceedings of the Royal Society B: Biological Sciences **268**:1337-1344.

Burger, H. 2002. Androgen production in women. Fertility and Sterility **77**:s3-s5.

Burke, C., and F. Roulet. 1970. Increased exposure of tissues to cortisol in late pregnancy. British Medical Journal **1**:657-659.

Burns, C., K. Rosvall, T. Hahn, G. Demas, and E. Ketterson. 2014. Examining sources of variation in HPG function among individuals and populations of the dark-eyed junco. Hormones and Behavior **65**:179-187.

Butler, D. 2009. asreml: asreml() fits the linear mixed model.

Calisi, R., and G. Bentley. 2009. Lab and field experiments: are they the same animal? Hormones and Behavior **56**:1-10.

Caramaschi, D., L. Booij, A. Petitclerc, M. Boivin, and R. Tremblay. 2012. Genetic and environmental contributions to saliva testosterone levels in male and female infant twins. Psychoneuroendocrinology **37**:1954-1959.

Carlsen, S., G. Jacobsen, and P. Romundstad. 2006. Maternal testosterone levels during pregnancy are associated with offspring size at birth. European Journal of Endocrinology **155**:365-370.

Carroll, J., E. Berg, T. Strauch, M. Roberts, and H. Kattesh. 2006. Hormonal profiles, behavioural responses, and short-term growth performance after castration of pigs at three, six, nine, or twelve days of age. Journal of Animal Science **84**:1271-1278.

Catchpole, E., Y. Fan, B. Morgan, T. Clutton-Brock, and T. Coulson. 2004. Sexual dimorphism, survival and dispersal in red deer. Journal of Agricultural, Biological and Environmental Statistics **9**:1-26.

Cavigelli, S. 1999. Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, Lemur catta. Animal Behaviour **57**:935-944.

Charmantier, A., and D. Garant. 2005. Environmental quality and evolutionary potential: lessons from wild populations. Proceedings of the Royal Society B: Biological Sciences **272**:1415-1425.

Chelini, M., N. Souza, A. Rocha, E. Felippe, and C. Oliveira. 2005. Quantification of fecal estradiol and progesterone metabolites in Syrian hamsters (Mesocricetus auratus). Brazilian Journal of Medical and Biological Research **38**:1711-1717.

Christensen, L., and R. Gorski. 1978. Independent masculinization of neuroendocrine systems by intracerebral implants of testosterone of estradiol in the neonatal female rat. Brain Research **146**:325-340.

Civantos, E. 2002. Testosterone supplementation in juvenile Psammodromus algirus lizards: consequences for aggressiveness and body growth. Acta Ethologica **4**:91-95.

Clarke, I., R. Scaramuzzi, and R. Short. 1976. Effects of testosterone implants in pregnant ewes on their female offspring. Journal of Embryology and Experimental Morphology **36**:87-99.

Clements, M., T. Clutton-Brock, S. Albon, J. Pemberton, and L. Kruuk. 2011. Gestation length variation in a wild ungulate. Functional Ecology **25**:691-703.

Clotfelter, E., D. O'Neal, J. Gaudioso, J. Casto, I. Parker-Renga, E. Snajdr, D. Duffer, V. Nolan, and E. Ketterson. 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? Hormones and Behavior **46**:171-178.

Clutton-Brock, T., S. Albon, and R. Gibson. 1979. The logical stag: adaptive aspects of fighting in red deer (Cervus elaphus L.). Animal Behaviour **27**:211-225.

Clutton-Brock, T., S. Albon, and F. Guinness. 1981. Parental investment in male and female offspring in polygynous mammals. Nature **289**:487-489.

Clutton-Brock, T., F. Guinness, and S. Albon. 1982. Red Deer: Behavior and Ecology of Two Sexes. The University of Chicago Press, Chicago.

Clutton-Brock, T., F. Guinness, and S. Albon. 1983. The costs of reproduction to red deer hinds. Journal of Animal Ecology **52**:367-383.

Clutton-Brock, T., M. Major, S. Albon, and F. Guinness. 1987. Early development and population dynamics in red deer. I. Density-dependent effects on juvenile survival. Journal of Animal Ecology **56**:53-67.

Clutton-Brock, T., and J. Pemberton. 2004. Soay Sheep: Dynamics and Selection in an Island Population. Cambridge University Press, Cambridge.

Clutton-Brock, T., and B. Sheldon. 2010. Individuals and populations: the role of long-term, individual-based studies of animals in ecology and evolutionary biology. Trends in Ecology and Evolution **25**:562-573.

Conradt, L., T. Clutton-Brock, and F. Guinness. 2000. Sex differences in weather sensitivity can cause habitat segrgation: red deer as an example. Animal Behaviour **59**:1049-1060.

Cook, C. 1997. Oxytocin and prolactin suppress cortisol responses to acute stress in both lactating and non-lactating sheep. Journal of Dairy Research **64**:327-339.

Cook, R., J. Cook, R. Garrott, L. Irwin, and S. Monfort. 2002. Effects of diet and body condition on fecal progestagen excretion in elk. Journal of Wildlife Diseases **38**:558-565.

Cooke, S., S. Hinch, G. Crossin, D. Patterson, K. English, H. Shrimpton, G. Van Der Kraak, and A. Farrell. 2006. Physiology of individual late-run Fraser River sockeye salmon (Oncorhynchus nerka) sampled in the ocean correlates with fate during spawning migration. Canadian Journal of Fisheries and Aquatic Science **63**:1469-1480.

Cooper, C., A. Evans, S. Cook, and N. Rawlings. 1995. Cortisol, progesterone and B-endorphin response to stress in calves. Canadian Journal of Animal Science **95**:197-201.

Corbier, P., D. Edwards, and J. Roffi. 1992. The neonatal testosterone surge- a comparative study. Archives Internationales De Physiologie De Biochimie Et De Biophysique **100**:127-131.

Corbier, P., J. Roffi, and J. Rhoda. 1983. Female sexual behavior in male rats: effect of hour of castration at birth. Physiology and Behavior **30**:613-616.

Coulson, T., S. Albon, F. Guinness, J. Pemberton, and T. Clutton-Brock. 1997. Population substructure, local density, and calf winter survival in red deer (Cervus elaphus). Ecology:852-863.

Coulson, T., S. Albon, J. Pemberton, J. Slate, F. Guinness, and T. Clutton-Brock. 1998. Genotype by environment interactions in winter survival in red deer. Journal of Animal Ecology **67**:434-445.

Coulson, T., F. Guinness, J. Pemberton, and T. Clutton-Brock. 2004. The demographic consequences of releasing a population of red deer from culling. Ecology **85**:411-422.

Coulson, T., L. Kruuk, G. Tavecchia, J. Pemberton, and T. Clutton-Brock. 2003. Estimating selection on neonatal traits in red deer using elasticity path analysis. Evolution **57**:2879-2892.

Coviello, A., W. Zhuang, K. Lunetta, S. Bhasin, J. Ulloor, A. Zhang, D. Karasik, D. Kiel, R. Vasan, and J. Murabito. 2011. Circulating testosterone and SHBG concentrations are heritable in women: the Framingham Heart Study. Journal of Clinical Endocrinology and Metabolism **96**:E1491-1995.

Cox, R., S. Skelly, and H. John-Alcer. 2005. Testosterone inhibits growth in juvenile male eastern fence lizards (Sceloporus undulatus): implications for energy allocation and sexual size dimorphism. Physiological and Biochemical Zoology **78**:531-545.

Cumming, D., M. Quigley, and S. Yen. 1983. Acute suppression of circulating testosterone levels by cortisol in men. The Journal of Clinical Endocrinology and Metabolism **57**:671-673.

Dachir, S., T. Kadar, B. Robinzon, and A. Levy. 1993. Cognitive deficits induced in young rats by long-term corticosterone administration. Behavioral and Neural Biology **60**:103-109.

Davison, S., R. Bell, S. Donath, J. Montalto, and S. Davis. 2005. Androgen levels in adult females: changes with age, menopause and oophorectomy. The Journal of Clinical Endocrinology and Metabolism **90**:3847-3853.

Decristophoris, P., A. von Hardenberg, and A. McElligott. 2007. Testosterone is positively related to the output of nematode eggs in male alpine ibex (Capra ibex) faeces. Evolutionary Ecology Research **9**:1277-1292.

Demisch, K., J. Grant, and W. Black. 1968. Plasma testoterone in women in late pregnancy and after delivery. Journal of Endocrinology **42**:477-481.

Despres, G., N. Rigaudiere, and P. Delost. 1984. Transfer of maternal testosterone to the guinea pig fetus during the last third of pregnancy. Biology of Reproduction **31**:7-15.

Deviche, P., L. Hurley, B. Fokidis, B. Lerbour, B. Silverin, B. Silverin, J. Sabo, and P. Sharp. 2010. Acute stress rapidly decreases plasma testosterone in a free-ranging male songbird: potential site of action and mechanism. General and Comparative Endocrinology **169**:82-90.

Deviche, R., S. Gao, S. Davies, P. Sharp, and A. Dawson. 2012. Rapid stress-induced inhibition of plasma testosterone in free-ranging male rufous-winged sparros, Peucaea carpalis: characterization, time course, and recovery. General and Comparative Endocrinology **177**:1-8.

Dey, C., C. O'Connor, K. Gilmour, G. Van Der Kraak, and S. Cooke. 2010. Behavioral and physiological responses of a wild teleost fish to cortisol and androgen manipulation during parental care. Hormones and Behavior **58**:599-605.

Diverio, S., P. Goddard, and I. Gordon. 1996. Use of long-acting neuroleptics to reduce the stress response to management practices in red deer. Applied Animal Behaviour Science **49**:83-88.

Dobson, H., and R. Smith. 1995. Stress and reproduction in farm animals. Journal of Reproduction and Fertility Suppliment **49**:451-461.

Elofsson, U., I. Mayer, B. Damsgard, and S. Winberg. 2000. Intermale competition in sexually mature Arctic charr: effects on brain monoamines, endocrine stress responses, sex hormone levels, and behavior. General and Comparative Endocrinology **118**:450-460.

Escribano-Avila, G., N. Pettorelli, E. Virgos, C. Lara-Romero, J. Lozano, I. Barja, F. Cuadra, and M. Puetra. 2013. Testing cort-fitness and cort-adaptation hypotheses in a habitat suitability gradient for roe deer. Acta Oecologica **53**:38-48.

Evans, M., M. Roberts, K. Buchanan, and A. Goldsmith. 2006. Heritability of corticosterone response and changes in life history traits during selection in the zebra finch. Journal of Evolutionary Biology **19**:343-352.

Evans, R. 1988. The steroid and thyroid-hormone receptor superfamily. Science **240**:889-895.

Falconer, D., and T. Mackay. 1996. Introduction to Quantitative Genetics. Longman, England.

Fletcher, T. 1978. The induction of male sexual behavior in red deer (Cervus elaphus) by the administration of testosterone to hinds and estradiol-17B to stags. Hormones and Behavior **11**:74-88.

Flood, J., S. Farr, F. Kaiser, M. Regina, and J. Morley. 1995. Age-related decrease of plasma testosterone in SAMP8 mice: replacement improves age-related impairment of learning and memory. Physiology and Behavior **57**:669-673.

Foecking, E., M. Szabo, N. Schwartz, and J. Levine. 2005. Neuroendocrine consequences of prenatal androgen exposure in the female rat: Absence of luteinizing hormone surges, suppression of progesterone receptor gene expression, and acceleration of the gonadotropin-releasing hormone pulse generator. Biology of Reproduction **72**:1475-1483.

Foerster, K., T. Coulson, B. Sheldon, J. Pemberton, T. Clutton-Brock, and L. Kruuk. 2007. Sexually antagonistic genetic variation for fitness in red deer. Nature **447**:1107-1110.

Folstad, I., and A. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. American Naturalist **139**:603-622.

Franks, S., L. Webber, M. Goh, A. Valentine, D. White, G. Conway, S. Wiltshire, and M. McCarthy. 2008. Ovarian morphology is a marker ofheritable biochemical traits in sisters with polycystic ovaries. Journal of Clinical Endocrinology and Metabolism **93**:3396-3402.

Gaiani, R., F. Chiesa, M. Mattioli, G. Nannetti, and G. Galeati. 1984. Androstenedione and testosterone concentrations in plasma and milk of the cow throughout pregnancy. Journal of Reproduction and Fertility **70**:55-59.

Ganswindt, A., M. Heistermann, S. Borragan, and J. Hodges. 2002. Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens Zoo Biology **21**:27-36.

Gerlach, N., and E. Ketterson. 2013. Experimental elevation of testosterone lowers fitness in female dark-eyed juncos. Hormones and Behavior **63**:782-790.

Gibson, R., and F. Guinness. 1980. Differential reproductive success in red deer stags. Journal of Animal Ecology **49**:199-208.

Gomendio, M., T. Clutton-Brock, S. Albon, F. Guinness, and M. Simpson. 1990. Mammalian sex ratios and variation in costs of rearing sons and daughters. Nature **343**:261-263.

Gonzalez-Parra, S., J. Argente, L. Garcia-Geura, and J. Chowen. 2000. Effect of neonatal and adult testosterone treatment on the cellular composition of the adult female rat anterior pituitary. Journal of Endocrinology **164**:265-276.

Good, T., M. Khan, and J. Lynch. 2003. Biochemical and physiological validation of a corticosteroid radioimmunoassay for plasma and fecal samples in oldfield mice (Peromyscus polionotus). Physiology and Behavior **80**:405-411.

Goymann, W. 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods in Ecology and Evolution **3**:757-765.

Goymann, W., M. Trappschuh, W. Jensen, and I. Schwabl. 2006. Low ambient temperature increases food intake and dropping production, leading to incorrect estimates of hormone metabolite concentrations in European stonechats. Hormones and Behavior **49**:644-653.

Graf, B., and M. Senn. 1999. Behavioural and physiological responses of calves to dehorning by heat cauterization with or without local anaesthesia. Applied Animal Behaviour Science **62**:153-171.

Graham, A., A. Hayward, K. Watt, J. Pilkington, J. Pemberton, and D. Nussey. 2010. Fitness correlates of heritable variation in antibody responsiveness in a wild mammal. Science **330**:662-665.

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

Greenberg, N., J. Carr, and C. Summers. 2002. Causes and consequences of stress. Integrative and Comparative Biology **42**:508-516.

Grenfell, B., O. Price, S. Albon, and T. Clutton-Brock. 1992. Overcompensation and population cycles in an ungulate. Nature **355**:823-826.

Groothuis, T., and H. Schwabl. 2008. Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? Philosophical Transactions of the Royal Society B: Biological Sciences **363**:1647-1661.

Groschl, M. 2008. Current status of salivary hormone analysis. Clinical Chemistry **54**:1759-1769.

Hadfield, J., D. Richardson, and T. Burke. 2006. Towards unbiased parentage assignment: combining genetic, behavioural and spatial data in a Bayesian framework. Molecular Ecology **15**:3715-3730.

Halbreich, U., G. Asnis, B. Zumoff, R. Nathan, and R. Shindledecker. 1984. Effect of age and sex on cortisol secretion in depressives and normals. Psychiatry Research **13**:221-229.

Hansen, P. 2009. Effects of heat stress on mammalian reproduction. Philosophical Transactions of the Royal Society B: Biological Sciences **364**:3341-3350.

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

Hau, M. 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. Bioessays **29**:133-144.

Hay, M., and P. Mormede. 1998. Urinary excretion of catecholamines, cortisol and their metabolites in Meishan and Lage White sows: validation as a non-invasive and integrative assessment of adrenocortical and symphatoadrenal axis activity. Veterinary Research **29**:119-128.

Heidinger, B., O. Chastel, I. Nisbet, and E. Ketterson. 2010. Mellowing with age: older parents are less responsive to a stressor in a long-lived seabird. Functional Ecology **24**:1037-1044.

Hews, D., and M. Moore. 1995. Influence of androgens on differentiation of secondary sex characteristics in tree lizards, Urosaurus ornatus. General and Comparative Endocrinology **97**:86-102.

Hill, P., R. Chatterton, and J. Aldag. 2003. Neuroendocrine responses to stressors in lactating and nonlactating mammals: a literature review. Biological Research for Nursing **5**:79-86.

Hiller-Sturmhofel, S. 1998. The endocrine system: an overview. Alcohol, Health and Research **22**:153-164.

Hirschenhauser, K., H. Winkler, and R. Oliveira. 2003. Comparative analysis of male androgen responsiveness to social environment in birds: the effects of mating system and paternal incubation. Hormones and Behavior **43**:508-519.

Hoby, S., F. Schwarzenberger, M. Doherr, N. Robert, and C. Walzer. 2006. Steroid hormone related male biased parasitism in chamois, Rupicapra rupicapra rupicapra. Veterinary Parasitology **138**:337-348.

Hoekstra, R., M. Bartels, and D. Boomsma. 2006. Heritability of testosterone levels in 12-year-old twins and its relation to pubertal development. Twin Research and Human Genetics **9**:558-565.

Hoffmann, B., and G. Schuler. 2002. The bovine placenta; a source and target of steroid hormones: observations during the second half of gestation. Domestic Animal Endocrinology **23**:309-320.

Hong, Y., J. Gagnon, T. Rice, L. Perusse, A. Leon, J. Skinner, J. Wilmore, C. Bouchard, and D. Rao. 2001. Familial resemblance for free androgens and androgen glucuronides in sedentary black and white individuals: the HERITAGE Family Study. Journal of Endocrinology **170**:485-492.

Howie, P., A. NcNeilly, M. Houston, A. Cook, and H. Boyle. 1981. Effects of supplementary food on suckling patterns and ovarian activity during lactation. British Medical Journal **283**:757-759.

Huber, S., R. Palme, and W. Arnold. 2003a. Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (Cerbus elaphus). General and Comparative Endocrinology **130**:48-54.

Huber, S., R. Palme, W. Zenker, and E. Mostl. 2003b. Non-invasive monitoring of the adrenocortical response in red deer. Journal of Wildlife Management **67**:258-266.

Huxley, J. 1932. Problems of Relative Growth. Methuen, London.

Ingram, J., J. Crockford, and L. Matthews. 1999. Ultradian, circadian and seasonal rhythms in cortisol secretion and adrenal responsiveness to ACTH and yarding in unrestrained red deer (Cervus elaphus) stags. Journal of Endocrinology **162**:289-300.

Jawor, J., J. McGlothlin, J. Casto, T. Greives, E. Snajdr, G. Bentley, and E. Ketterson. 2006. Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (Junco hyemalis). General and Comparative Endocrinology **149**:182-189.

Jessop, T., and M. Hamann. 2005. Interplay between age class, sex, and stress response in green turtles (Chelonia mydas). Australian Journal of Zoology **2**:131-136.

Jones, M., E. Folkerd, D. Doody, J. Iqbal, M. Dowsett, A. Ashworth, and A. Swerdlow. 2007. Effects of delays in processing blood samples on measured endogenous plasma sex hormone levels in women. Cancer Epidemiology, Biomarkers and Prevention **16**:1136-1139.

Kaiser, S., and N. Sachser. 2005. The effects of prenatal social stress on behaviour: mechanisms and function. Neuroscience and Biobehavioural Reviews **29**:283-294.

Karsch, F. 1987. Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. Annual Reviews in Physiology **49**:364-382.

Karsch, F., J. Robinson, C. Woodfill, and M. Brown. 1989. Circannual cycles of luteinizing hormone and prolactin secretion in ewes during prolonged exposure to a fixed photoperiod: evidence for an endogenous reproductive rhythm. Biology and Reproduction **41**:1034-1046.

Kempenaers, B., A. Peters, and K. Foerster. 2008. Sources of individual variation in plasma testosterone levels. Philosophical Transactions of the Royal Society B: Biological Sciences **363**:2722-2723.

Kent, J., V. Molony, and I. Robertson. 1993. Changes in plasma cortisol concentration in lambs of three ages after three methods of castration and tail docking. Research in Veterinary Science **55**:246-251.

Ketterson, E., V. Nolan Jr, and M. Sandell. 2005. Testosterone in females: mediator of adaptive traits, constraint on sexual dimorphism, or both? The American Naturalist **166**:s85-s95.

Ketterson, E., V. Nolan, L. Wolf, C. Ziegenfus, A. Dufty, G. Ball, and T. Johnsen. 1991. Testosterone and avian life histories: the effect of experimentally elevated testosterone on corticosterone and body mass in dark-eyed juncos. Hormones and Behavior **25**:489-503.

Khatri, P. 2011. The bovine placenta as a source and target of steroid hormones: aspects on the role of androgens and sulfonated steroids. Justus Liebig University, Giessen, Germany.

King, R., J. Cline, and C. Hubbard. 2004. Heritabile variation in testosterone levels in male garter snakes. Journal of Zoology **264**:143-147.

Klasing, K. 2005. Potential impact of nutritional strategy on noninvasive measurements of hormones in birds. Annals of the New York Academy of Sciences **1046**:5-16.

Koren, L., O. Mokady, T. Karaskov, J. Klein, G. Koren, and E. Geffen. 2002. A novel method using hair for determining hormonal levels in wildlife. Animal Behaviour **61**:401-406.

Kralj-Fisher, S., I. Scheiber, A. Blejec, E. Moestl, and K. Kotrschal. 2007. Individualities in a flock of freeroaming greylag geese: behavioral and physiological consistency over time and across situations. Hormones and Behavior **51**:239-248.

Kruuk, L. 2004. Estimating genetic parameters in natural animal populations using the "animal model". Philosophical Transactions of the Royal Society B: Biological Sciences **359**:873-890.

Kruuk, L., and J. Hadfield. 2007. How to separate genetic and environmental causes of similarity between relatives. Journal of Evolutionary Biology **20**:1890-1903.

Kuijper, E., C. Lambalk, D. Boomsma, S. van der Sluis, M. Blankenstein, E. de Geus, and D. Posthuma. 2007. Heritability of reproductive hormones in adult male twins. Human Reproduction **22**:2153-2159.

Lande, R. 1987. Genetic correlations between the sexes in the evolution of sexual dimorphism and mating preferences.*in* J. A. Bradbury, M., editor. Sexual Selection: Testing the Alternatives. John Wiley & Sons Ltd.

Landete-Castillejos, T., A. Garcia, F. Lopez-Serrano, and L. Gallego. 2005. Maternal quality and differences in milk production and composition for male and female Iberian red deer calves (Cervus elaphus hispanicus). Behavioral Ecology and Sociobiology **57**:267-274.

Landete-Castillejos, T., C. Gortazar, J. Vicente, Y. Fierro, A. Garcia, and L. Gallego. 2004. Age-related foetal sex ratio bias in Iberian red deer (Cervus elaphus hispanicus): are male calves too expensive for growing mothers? Behavioral Ecology and Sociobiology **56**:1-8.

Langvatn, R., S. Albon, T. Burkey, and T. Clutton-Brock. 1996. Climate, plant phenology and variation in age of first reproduction in a temperate herbivore. Journal of Animal Ecology **65**:653-670.

Lank, D., M. Coupe, and K. Wynne-Edwards. 1999. Testosterone-induced male traits in female ruffs (Philomachus pugnax): autosomal inheritance and gender differentiation. Proceedings of the Royal Society B: Biological Sciences **266**:2323-2330.

Lee, V., D. Dekretser, B. Hudson, and C. Wang. 1975. Variations in serum FSH, LH and testosterone levels in male rats from birth to sexual maturity. Journal of Reproduction and Fertility **42**:121-126.

Lemaitre, J., C. Vanpe, F. Plard, and J. Gaillard. 2014. The allometry between secondary sexual traits and body size is nonlinear amongst cervids. Biology Letters **10**.

Licht, P., L. Frank, S. Pavgi, T. Yalcinkaya, P. Siiteri, and S. Glickman. 1992. Hormonal correlates of "masculinization" in female spotted hyaenas (Crocuta crocuta). 2. Maternal and fetal steroids. Journal of Reproduction and Fertility **95**:463-374.

Licht, P., T. Hayes, P. Tsai, G. Cuhna, H. Kim, M. Golbus, S. Hayward, M. Martin, R. Jaffe, and S. Glickman. 1998. Androgens and masculinzation of genitalia in the female spotted hyaena (Crocuta crocuta). 1. Urogenital morphology and placental androgen production during fetal life. Journal of Reproduction and Fertility **133**:105-116.

Lincoln, G. 1971. The seasonal reproductive changes in the red deer stag (Cervus elaphus). Journal of Zoology **163**:105-123.

Lincoln, G. 1972. Role of antlers in behavior of red deer. Journal of Experimental Zoology **182**:233-&.

Lincoln, G. 1973. Appearance of antler pedicles in early foetal life in red deer. Journal of Embryology and Experimental Morphology **29**:431-437.

Lincoln, G., F. Guinness, and R. Short. 1972. Way in which testosterone controls social and sexual-behavior of red deer stag (Cervus elaphus). Hormones and Behavior **3**:375-396.

Lincoln, G., and R. Kay. 1979. Effects of season on the secretion of LH and testosterone in intact and castrated red deer stags (Cervus elaphus). Journal of Reproduction and Fertility **55**:75-80.

Liptrap, R. 1993. Stress and reproduction in domestic animals. Annals of the New York Academy of Science **697**:275-284.

Lopez-Olivera, J., I. Marco, J. Montane, E. Casas-Diaz, and S. Lavin. 2007. Effects of acepromazine on the stress response in Southern chamois (Rupicapra pyrenaica) captured by means of drive-nets. Canadian Journal of Veterinary Research **71**:41-51.

Loudon, A., and P. Racey. 1987. Reproductive Energetics in Mammals. Oxford University Press, New York.

Lovari, S., J. Herrero, J. Conroy, T. Maran, G. Giannatos, M. Stubbe, S. Aulagnier, T. Jdeidi, M. Masseti, I. Nader, K. de Smet, and F. Cuzin. 2008. Cervus elaphus. IUCN Red List of Threatened Species.

Lowe, V. 1961. A discussion on the history, present status and future conservation of the red deer (Cervus elaphus L.) in Scotland. Terre et Vie **1**:9-40.

Lubritz, D., B. Johnson, and O. Robinson. 1991. Genetic parameters for testosterone production in boars. Journal of Animal Science **69**:3220-3224.

Lummaa, V., J. Pettay, and A. Russell. 2007. Male twins reduce fitness of female co-twins in humans. Proceedings of the National Academy of Sciences **104**:10915-10920.

Lynch, J., T. Ziegler, and K. Strier. 2002. Individual and seasonal variation in fecal testosterone and cortisol levels of wild male tufted capuchin monkeys, Cebus apella nigritus. Hormones and Behavior **41**:275-287.

Lynch, M., and B. Walsh. 1998. Genetics and Analysis of Quantitative Traits. Sinauer, Sunderland, Massachusetts.

Maccoby, E., C. Doering, C. Jacklin, and H. Kraemer. 1979. Concentrations of sex hormones in the umbilical-cord: their relation to sex and birth order of infants. Child Development **50**:632-642.

Mackinnon, M., N. Corbet, K. Meyer, H. Burrow, R. Bryan, and D. Hetzel. 1991. Genetic parameters for testosterone response to GnRH stimulation and scrotal circumference in tropical beef bulls. Livestock Production Science **29**:297-309.

Macrides, F., A. Bartke, F. Fernandez, and W. D'Angelo. 1974. Effects of exposure to vaginal odor and receptive females on plasma testosterone in the male hamster. Neuroendocrinology **15**:355-364.

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

Malpaux, B. 2006. Seasonal regulation of reproduction in mammals. Pages 2231-2281 *in* J. Neill, editor. Knobil and Neill's Physiology of Reproduction. Elsevier, St. Louis, Missouri.

Mank, J. 2007. The evolution of sexually selected traits and antagonistic androgen expression in Actinopterygiian fishes. The American Naturalist **169**:142-149.

Mann, D., and H. Fraser. 1996. The neonatal period: A critical interval in male primate development. Journal of Endocrinology **149**:191-197.

Marler, C. A., and M. C. Moore. 1988. Evolutionary costs of aggression revealed by testosterone manipulations in free-living male lizards. Behavioral Ecology and Sociobiology **23**:21-26.

Mason, W., S. Mendoza, and R. Sapolsky. 1993. The physiology of dominance in stable versus unstable social hierarchies. Pages 171-204 Primate Social Conflict. State University of New York Press, Albany.

Matuszczyk, J., B. Silverin, and K. Larsson. 1990. Influence of environment events immediately after brith on postnatal testosterone secretion and adult sexual behavior in the male rat. Hormones and Behavior **24**:450-458.

McDonald, P., and C. Doughty. 1972. Comparison of the effect of neonatal administration of testosterone and dihydrotestosterone in the female rat. Journal of Reproduction and Fertility **30**:55-62.

McMeekan, C., D. Mellor, K. Stafford, R. Bruce, R. Ward, and N. Gregory. 1997. Effects of shallow scoop and deep scoop dehorning on plasma cortisol concentrations in calves. New Zealand Veterinary Journal **45**:72-74.

McMillen, I., G. Thorburn, and D. Walker. 1987. Diurnal variations in plasma concentrations of cortisol, prolactin, growth hormones and glucose in the fetal sheep and pregnant ewe during late gestation. Journal of Endocrinology **114**:65-72.

Meikle, A., D. Bishop, J. Stringham, and D. West. 1986. Quantitating genetic and non genetic factors that determine plasma sex steroid variation in normal male twins. Metabolism **35**:1090-1095.

Meikle, A., J. Stringham, M. Woodward, and D. Bishop. 1988. Heritability of variation of plasma cortisol levels. Metabolism **37**:514-517.

Meulenberg, P., and J. Hofman. 1991. Maternal testosterone and fetal sex. The Journal of Steroid Biochemistry and Molecular Biology **39**:51-54.

Michel, C., O. Chastel, and X. Bonnet. 2011. Ambient temperature and pregnancy influence cortisol levels in female guinea pigs and entail long-term effects on the stress response of their offspring. General and Comparative Endocrinology **171**:275-282.

Mills, S., A. Grapputo, I. Jokinen, E. Koskela, T. Mappes, T. Oksanen, and T. Poikonen. 2009. Testosterone-mediated effects on fitness-related phenotypic traits and fitness. The American Naturalist **173**:475-487.

Mills, S., E. Koskela, and T. Mappes. 2012. Intralocus sexual conflict for fitness: sexually antagonistic alleles for testosterone. . Proceedings of the Royal Society B: Biological Sciences **279**:1889-1895.

Millspaugh, J., and B. Washburn. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. General Comparative Endocrinology **138**:189-199.

Moberg, G. 2000. Biological responses to stress. Pages 1-21 The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Pub., Wallingford, New York.

Mohle, U., M. Heistermann, R. Palme, and J. Hodges. 2002. Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing

gonadal endocrine function in male nonhuman primates. General and Comparative Endocrinology **129**:135-145.

Mokkonen, M., E. Koskela, T. Mappes, and S. Mills. 2012. Sexual antagonism for testosterone maintains multiple mating behaviour. Animal Behaviour **81**:277-283.

Moller, A., L. Garamszegi, D. Gil, S. Hurtrez-Bousses, and M. Eens. 2005. Correlated evolution of male and female testosterone profiles in birds and its consequences. Behavioral Ecology and Sociobiology **58**:534-544.

Mondragon, J., R. Valdez, Y. Gomez, A. Rosales, and M. Romano. 2012. Study of the steroidogenic pathways involved in goat placental androgen and estrogen synthesis. Small Ruminant Research **106**:173-177.

Montane, J., I. Markco, J. Lopez-Olivera, D. Perpinan, X. Manteca, and S. Lavin. 2003. Effects of acepromazine on capture stress in roe deer (Capreolus capreolus). Journal of Wildlife Diseases **39**:375-386.

Moore, M., C. Thompson, and C. Marler. 1991. Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, Urosaurus ornatus. General and Comparative Endocrinology **81**:217-226.

Morrow, C., and S. Monfort. 1998. Ovarian activity in the scimitar-horned oryx (Oryx dammah) determined by faecal steroid analysis. Animal Reproduction Science **53**:191-207.

Mostl, E., S. Messmann, E. Bagu, C. Robia, and R. Palme. 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. Journal of Veterinary Medicine A **46**:621-631.

Mostl, E., and R. Palme. 2002. Hormones as indicators of stress. Domestic Animal Endocrinology **23**:67-74.

Motelica-Heino, I., D. Edwards, and J. Roffi. 1993. Intermale aggression in mice: does hour of castration after birth influence adult behavior? Physiology and Behavior **53**:1017-1019.

Mougeot, F., L. Perez-Rodriguez, J. Martinez-Padilla, F. Leckie, and S. Redpath. 2007. Parasites, testosterone an honest carotenoid-based signalling of health. Functional Ecology **21**:886-898.

Moyes, K., T. Coulson, B. Morgan, A. Donald, S. Morris, and T. Clutton-Brock. 2006. Cumulative reproduction and survival costs in female red deer. Oikos **115**:241-252.

Muller, M., and R. Wrangham. 2004a. Dominance, aggression and testosterone in wild chimpanzees: a test of he 'challenge hypothesis'. Animal Behaviour **67**:113-123.

Muller, M., and R. Wrangham. 2004b. Dominance, cortisol and stress in wild chimpanzees (Pan troglodytes schweinfurthii). Behaviour, Ecology and Sociobiology **55**:332-340.

Muller, W., T. Groothuis, C. Eising, and C. Dijkstra. 2005. An experimental study on the causes of sex-biased mortality in the black-headed gull- the possible role of testosterone. Ecology **74**:735-741.

Musey, V., D. Collins, D. Brogan, V. Santos, P. Musey, D. Martino-Saltzman, and J. Preedy. 1987. Long term effects of a first pregnancy on the hormonal environment: estrogens and androgens. Journal of Clinical Endocrinology and Metabolism **64**:111-118.

Mysterud, A., N. Yoccoz, R. Langvatn, N. Pettorelli, and N. Stenseth. 2008. Hierarchical path analysis of deer responses to direct and indirect effects of climate in northern forest. Philosophical Transactions of the Royal Society B: Biological Science **363**:2359-2368.

Nakada, K., M. Moriyoshi, T. Nakao, G. Watanabe, and K. Taya. 2000. Changes in concentrations of plasma immunoreactive follicle-stimulating hormone, luteinizing hormone, estradiol-17 beta, testosterone, progesterone, and inhibin in heifers from birth to puberty. Domestic Animal Endocrinology **18**:57-69.

Nussey, D., L. Kruuk, A. Donald, M. Fowlie, and T. Clutton-Brock. 2006. The rate of senescence in maternal performance increases with early-life fecundity in red deer. Ecology Letters **9**:1342-1350.

Nussey, D., L. Kruuk, A. Morris, M. Clements, J. Pemberton, and T. Clutton-Brock. 2009. Inter- and intrasexual variation in aging patterns across reproductive traits in a wild red deer population. The American Naturalist **174**:342-357.

Nyirenda, M., R. Lindsay, C. Kenyon, A. Burchell, and J. Secjl. 1998. Glucocorticoid exposure in late gestation permanently programmes rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. Journal of Clinical Investigation **101**:2174-2181.

O'Connor, C., K. Gilmour, R. Arlinghaus, G. Van Der Kraak, and S. Cooke. 2009. Stress and parental care in a wild teleost fish: insights from exogenous supraphysiological cortisol implants. Physiological and Biochemical Zoology **82**:709-719.

Obel, C., M. Hedegaard, T. Henriksen, N. Secher, J. Olsen, and S. Levine. 2005. Stress and salivary cortisol during pregnancy. Psychoneuroendocrinology **30**:647-656.

Okuliarova, M., T. Groothuis, P. Skrobanek, and M. Zaman. 2011. Experimental evidence for genetic heritability of maternal hormone transfer to offspring. The American Naturalist **177**:824-834.

Oliveira, R. 2004. Social modulation of androgens in vertebrates: mechanisms and function. Advances in the Study of Behavior **34**:165-239.

Oliveira, R., M. Lopes, L. Carneiro, and A. Canario. 2001. Watching fights raises fish hormone levels. Nature **409**:475.

Olsson, M., E. Wapstra, T. Madsen, and B. Silverin. 2000. Testosterone, ticks and travels: a test of the immunocompetence-handicap hypothesis in free-ranging male sand lizards. Proceedings of the Royal Society of London B: Biological Sciences **267**:2339-2343.

Otte, C., S. Hart, T. Neylan, D. Marmar, K. Yaffe, and D. Mohr. 2005. A meta-analysis of cortisol response to challenge in human aging: importance of gender. Psychoneuroendocrinology **30**:80-91.

Owen-Ashley, N., D. Hasselquist, and J. Wingfield. 2004. Androgens and immunocompetence handicap hypothesis: unravelling direct and indirect pathways of immunosuppression in song sparrows. The American Naturalist **164**:490-505.

Palme, R. 2005. Measuring fecal steroids: guidelines for practical application. Annals of the New York Academy of Sciences **1046**:75-80.

Palme, R., and E. Mostl. 1994. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. Pages 111-117 *in* S. Gorog, editor. Advances in Steroid Analysis '93. Akademiai Kiado, Budapest.

Palme, R., S. Rettensacher, C. Touma, S. El-Bahr, and E. Mostl. 2005. Sress hormones in mammals and birds: comparative aspects regarding metabolism, excretion,

and noninvasive measurement in fecal samples. Trends in Comparative Endocrinology and Neurobiology **1040**:162-171.

Pankhurst, N., and P. Munday. 2011. Effects of climate change on fish reproduction and early life history stages. Marine and Freshwater Research **62**:1015-1026.

Parker, M., and R. Mason. 2012. How to make a sexy snake: estrogen activation of female sex pheromone in male red-sided garter snakes. Journal of Experimental Biology **1**:723-730.

Pavitt, A., C. Walling, E. Mostl, J. Pemberton, and L. Kruuk. In review. Cortisol but not testosterone is repeatable and varies with an index of reproductive effort in wild red deer stags. General and Comparative Endocrinology.

Pavitt, A., C. Walling, J. Pemberton, and L. Kruuk. 2014a. Causes and consequences of variation in early life testosterone in a wild population of red deer. Functional Ecology **28**:1224-1234.

Pavitt, A., C. Walling, J. Pemberton, and L. Kruuk. 2014b. Heritability and cross-sex genetic correlations of early-life circulating testosterone levels in a wild mammal. Biology Letters **10**.

Pavitt, A., C. Walling, J. Pemberton, and L. Kruuk. In prep. Testosterone and cortisol concentrations vary with reproductive status in wild female red deer. Oecologia.

Pelletier, F., J. Bauman, and M. Festa-Bianchet. 2003. Fecal testosterone in bighorn sheep (Ovis canadensis): behavioural and endocrine correlates. Canadian Journal of Zoology **81**:1678-1684.

Pemberton, J., S. Albon, F. Guinness, T. Clutton-Brock, and G. Dover. 1992. Behavioral estimates of male mating success tested by DNA fingerprinting in a polygynous mammal. Behavioral Ecology **3**:66-75.

Pereira, R., J. Duarte, and J. Negrao. 2005. Seasonal changes in fecal testosterone concentrations and their relationship to the reproductive behavior, antler cycle and grouping patterns in free-ranging male pampas deer (Ozotoceros bezoarticus bezoarticus). Theriogenology **63**:2113-2125.

Perez-Rodriguez, L., J. Blas, J. Vinuela, T. Marchant, and G. Bortolotti. 2006. Condition and androgen levels: are condition-dependent and testosterone-mediated traits two sides of the same coin? Animal Behaviour **72**:97-103.

Peters, A., L. Astheimer, and A. Cockburn. 2001. The annual testosterone profile in cooperative breeding superb fairy-wrens, Malurus cyaneus, reflecting their extreme infidelity. Behavioral Ecology and Sociobiology **50**:519-527.

Peterson, M., K. Rosvall, C. Taylor, J. Lopex, J. Choi, C. Ziegenfus, H. Tang, J. Colbourne, and E. Ketterson. 2014. Potential for sexual conflict assessed via testosterone-mediated transcriptional changes in liver and muscle of a songbird. Journal of Experimental Biology **217**:507-517.

Pfaff, D., and R. Zigmond. 1971. Neonatal androgen effects on sexual and non-sexual behavior of adult rats tested under various hormone regimes. Neuroendocrinology **7**:129-145.

Pfeiffer, C. 1936. Sexual differences of the hypophyses and their determination by the gonads. American Journal of Anatomy **58**:195-225.

Pinheiro, J., D. Bates, S. DebRoy, and D. Sarkar. 2014. nlme: linear and nonlinear mixed effects models.

Pinxten, R., E. de Ridder, L. Arckens, V. Darras, and M. Eens. 2007. Plasma testosterone lvels of male European starlings (Sturnus vulgaris) during the breeding cycle and in relation to song and paternal care. Behaviour **144**:393-410.

Pottinger, T., and T. Carrick. 1999. Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. General and Comparative Endocrinology **116**:122-132.

R.Development.Core.Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rabiee, A., K. Macmillan, and F. Schwarzenberger. 2001. The effect of level of feed intake on progesterone clearance rate by measuring faecal progesterone metabolites in grazing dairy cows. Animal Reproduction Science **67**:205-214.

Reed, W., M. Clark, P. Parker, S. Faouf, N. Arguedas, D. Monk, E. Snajdr, V. Nolan Jr, and E. Ketterson. 2006. Physiological effects on demography: a long term experimental study of testosterone's effects on fitness. The American Naturalist **167**:667-683.

Rickard, I., V. Lummaa, and A. Russell. 2009. Elder brothers affect the life history of younger siblings in preindustrial humans: social consequence or biological cost? Evolution and Human Behavior **30**:49-57.

Ring, H., C. Lessov, T. Reed, R. Marcus, L. Holloway, G. Swan, and D. Carmelli. 2005. Heritability of plasma sex hormones and hormone binding globulin in adult male twins. Journal of Clinical Endocrinology and Metabolism **90**:3653-3658.

Robinson, J., K. Sinclair, and T. McEvoy. 1999. Nutritional effects on foetal growth. Animal Science **68**:315-331.

Robinson, O., D. Lubritz, and B. Johnson. 1994. Realized heritability estimates in boars divergently selected for testosterone levels. Journal of Animal Breeding Genetics **111**:35-42.

Roff, D., and A. Simons. 1997. The quantitative genetics of wind dimorphism under laboratory and "field" conditions in the cricket Gryllus pennsylvanicus. Heredity **78**:235-240.

Roffi, J., F. Chami, P. Corbier, and D. A. Edwards. 1987. Testicular hormones during the first few hours after birth augment the tendency of adult male-rats to mount receptive females. Physiology and Behavior **39**:625-628.

Romero, L. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinology **128**:1-24.

Romero, L. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology and Evolution **19**:249-255.

Romero, L., and L. Butler. 2007. Endocrinology of stress. International Journal of Comparative Psychology **20**:89-95.

Romero, L., and J. Reed. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comparative Biochemistry and Physiology, Part A **140**:73-79.

Rosenblatt, J., C. Wagner, and J. Morrell. 1994. Hormonal priming and triggering of maternal behavior in the rate with species reference to the relationship between estrogen receptor binding and ER mRNA in specific brain regions. Psychoneuroendocrinology **19**:543-552.

Rosvall, K. 2013. Life history trade-offs and behavioral sensitivity to testosterone: an experimental test when female aggression and maternal care co-occur. 8.

Runfeldt, S., and J. Wingfield. 1985. Experimentally prolonged sexual activity in female sparrows delays termination of reproductive activity in their untreated males. Animal Behaviour **33**:403-410.

Rushen, J., T. Nay, L. Wright, D. Payne, and G. Foxcroft. 1995. Stress and nursing in the pig: role of HPA axis and endogenous opioid peptides. Physiology and Behavior **58**:43-48.

Ryan, C., G. Anderson, L. Gardiner, and J. Hare. 2012. Sex-induced sex ratios in ground squirrels: support for a mechanistic hypothesis. Behavioral Ecology **23**:160-167.

Saino, N., R. Ferrari, M. Romano, R. Martinelli, A. Lacroix, D. Gil, and A. Moller. 2006. Maternal allocation of androgens and antagonistic effects of yolk androgens on sons and daughters. Behavioral Ecology **17**:172-181.

Sakai, L., L. Baker, C. Jacklin, and I. Shulman. 1992. Sex steroids at birth: genetic and environmental variation and covariation. Developmental Psychobiology **24**:559-570.

Saltz, D., and G. White. 1991. Urinary cortisol and urea nitrogen responses to winter stress in mule deer. Journal of Wildlife Management **55**:1-16.

Sapolsky, R. 1986. Stress-induced elevation of testosterone concentration in high ranking baboons: role of catecholamines. Endocrinology **118**:1630-1635.

Sapolsky, R. 1991. Do glucocorticoid concentrations rise with age in the rat? Neurobiology of Aging **13**:171-174.

Sapolsky, R. 1992. Cortisol concentrations and the social significance of rank instability among wild baboons. Psychoneuroendocrinology **17**:701-709.

Sapolsky, R., L. Krey, and B. McEwen. 1984. Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response. Proceedings of the National Academy of Science USA **81**:6174-6177.

Sapolsky, R., L. Krey, and B. McEwen. 1986. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocrine Reviews **7**:284-301.

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

Sasvari, L., Z. Hegyi, and P. Peczely. 1999. Brood reduction in white stork mediated through asymmetries in plasma testosterone concentration in chicks. Ethology **105**:569-582.

Sasvari, L., Z. Hegyi, and P. Peczely. 2006. Maternal condition affects the offspring testosterone concentration in early nestling period: an experimental test on tawny owls. Ethology **112**:355-361.

Sathishkumar, K., R. Elkins, V. Chinnathambi, H. Gao, G. Hankins, and C. Yallampalli. 2011. Prenatal testosterone-induced fetal growth restriction is associated with down-regulation of rat placental amino acid transport. Reproductive Biology and Endocrinology **9**:110-122.

Schatz, S., and R. Palme. 2001. Measurement of faecal cortisol metabolites in cats and dogs: a non-invasive method for evaluating adrenocortical function. Veterinary Research Communications **25**:271-287.

Schroderus, E., I. Jokinen, M. Koivula, T. Mappes, S. Mills, T. Oksanen, and T. Poikonen. 2010. Intra- and intersexual trade-offs between testosterone and immune system: implications for sexual and sexually antagonistic selection. The American Naturalist **176**:E90-E97.

Schwabl, H. 1993. Yolk is a source of maternal testosterone for developing birds. Proceedings from the National Academy of Science **90**:446-450.

Schwarzenberger, F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. International Zoo Yearbook **41**:52-74.

Schwarzenberger, F., E. Mostl, R. Palme, and E. Bamberg. 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Animal Reproduction Science **42**:515-526.

Scott, E., H. McGarrigle, and G. Lachelin. 1990. The increase in plasma and saliva cortisol levels in pregnancy is not due to the increase in corticosteroid-binding globulin levels. Journal of Clinical Endocrinology and Metabolism **71**:639-644.

Seckl, J. 2004. Prenatal glucocorticoids and long-term programming. European Journal of Endocrinology **151**:U49-U62.

Seney, M., C. Walsh, R. Stolakis, and E. Sibille. 2012. Neonatal testosterone partially organizes sex differences in stress-induced emotionality in mice. Neurobiology of Disease **46**:486-496.

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

Sharma, I., S. Agarwal, V. Agarwal, and P. Dwaraknath. 1984. Changes in profiles of serum sex steroids of male buffaloes from birth to maturity. Theriogenology **22**:175-186.

Silberzahn, P., I. Zwain, and B. Martin. 1984. Concentration increase of unbound testosterone in plasma of the mare throughout pregnancy. Endocrinology **115**:416-419.

Sinclair, A., S. Mduma, J. Hopcraft, J. Fryxell, R. Hilborn, and S. Thirgood. 2007. Long-term ecosystem dynamics in the Serengeti: lessons for conservation. Conservation Biology **21**:580-590.

Slob, A., R. den Hamer, P. Woutersen, and J. Van der Werff ten Bosch. 1983. Prenatal testoserone propionate and postnatal ovarian activity in the rat. Acta Endocrinologica **103**:420-427.

Slob, A., M. Ooms, and J. Vreeburg. 1980. Prenatal and early postnatal sex differences in plasma and gonadal testosterone and plasma luteinizing hormone in female and male rats. Journal of Endocrinology **87**:81-87.

Sluyter, F., J. Keijser, D. Boomsma, L. van Doornen, E. van den Oord, and H. Snieder. 2000. Genetics of testosterone and the aggression-hostility-anger (AHA) syndrome: a study of middle-aged twins. Twin Research **3**:266-276.

Smith, C., and R. Norman. 1987. Influence of the gonads on cortisol secretion in female rhesus macaques. Endocrinology **121**:2192-2198.

SNH. 2009. The Story of Rum National Nature Reserve, www.snh.gov.uk.

Squires, E. 2003. Applied Animal Endocrinology. CABI Publishing, UK.

Staub, N., and M. DeBeer. 1997. The role of androgens in female vertebrates. General and Comparative Endocrinology **108**:1-24.

Stavisky, R., S. Watson, M. Anthony, S. Manuck, M. Adams, and J. Kaplan. 2003. Influence of estradiol on cortisol secretion in ovariectomized cynomolgus macaques (Macaca fascicularis). American Journal of Primatology **60**:17-22.

Stocco, D., and B. Clark. 1996. Regulation of the acute production of steroids in steroidogenic cells. Endocrine Reviews **17**:221-244.

Stone, J., E. Folkerd, D. Doody, C. Schroen, S. Treloar, G. Giles, M. Pike, D. English, M. Southey, J. Hopper, and M. Dowsett. 2009. Familial correlations in postmenopausal serum concentrations of sex steroid hormones and other

mitogens: a twins and sister study. Journal of Clinical Endocrinology and Metabolism **94**:4793-4800.

Stopher, K., A. Bento, T. Clutton-Brock, J. Pemberton, and L. Kruuk. 2014. Multiple pathways mediate effects of climate change on maternal reproductive traits in a red deer population. Ecology **95**:3124-3138.

Strasser, R., and H. Schwabl. 2004. Yolk testosterone organizes behaviour and male plumage coloration in house sparrows. Behavioral Ecology and Sociobiology **56**:491-497.

Strauss, J., F. Martinez, and M. Kiriakidou. 1996. Placental steroid hormone synthesis: Unique features and unanswered questions. Biology of Reproduction **54**:303-311.

Strier, K., T. Ziegler, and D. Wittwer. 1999. Seasonal and social correlates of fecal testosterone and cortisol levels in wild male muriquis (Brachyteles arachnoides). Hormones and Behavior **35**:125 -134.

Summers, C., M. Watt, T. Ling, G. Forster, R. Carpenter, W. Korzan, J. Lukkes, and O. Overli. 2005. Glucocorticoid interaction with aggression in non-mammalian vertebrates: reciprocal action. European Journal of Pharmacology **526**:21-35.

Sun, M., M. Maliqueo, A. Benrick, J. Johansson, R. Shao, L. Hou, T. Jansson, X. Wu, and E. Stener-Victorin. 2012. Maternal androgen excess reduced placental and fetal weights, increases placental steroidogenesis, and leads to long-term health effects in their female offspring. American Journal of Physiology: Endocrinology and Metabolism **303**:E1373-E1385.

Suttie, J., P. Fennessy, I. Corson, F. Laas, H. Elgar, and K. Lapwood. 1989. LH and testosterone responses to GNRH in red deer (Cervus elaphus) stags kept in manipulated photoperiod. Journal of Reproduction and Fertility **85**:213-219.

Suttie, J., P. Fennessy, I. Corson, B. Veenvliet, R. Littlejohn, and K. Lapwood. 1992. Seasonal pattern of luteinizing hormone and testosterone pulsatile secretion in young adult red deer stags (Cervus elaphus) and its association with the antler cycle. Journal of Reproduction and Fertility **95**:925-933.

Suttie, J., G. Lincoln, and R. Kay. 1984. Endocrine control of antler growth in red deer stags. Journal of Reproduction and Fertility **71**:7-15.

Taitt, M., and C. Krebs. 1982. Manipulation of female behaviour in field populations of Microtus townsendii. Journal of Animal Ecology **51**:681-690.

Tapanainen, J. 1983. Hormonal changes during the perinatal period: serum testosterone, some of its precursors, and FSH and prolactin in preterm and full-term male infant cord blood and during the first week of life. Journal of Steroid Biochemistry and Molecular Biology **18**:13-18.

Terio, K., L. Marker, and L. Munson. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (Acinonyx jubatus) based on adrenal morphology and function. Journal of Wildlife Diseases **40**:259-266.

Teskey-Gerstl, A., T. Steineck, E. Bamberg, and R. Palme. 2000. Excretion of corticosteroids in urine and faeces of hares (Lepus europaeus). Journal of Comparative Physiology B **170**:163-168.

Thoman, E., R. Conner, and S. Levin. 1970. Lactation suppresses adrenal corticosteroid activity and aggressiveness in rats. Journal of Comparative and Physiological Psychology **70**:364-369.

Touma, C., and R. Palme. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of a biological validation. Annals of the New York Academy of Sciences **1046**:54-74.

Travison, T., W. Zhuang, K. Lunetta, D. Karasik, S. Bhasin, D. Kiel, A. Coviello, and J. Murabito. 2014. The heritability of circulating testosterone, oestradiol, oestrone and sex hormone binding globulin concentrations in men: the Framingham Heart Study. Clinical Endocrinology **80**:277-282.

Tschirren, B., J. Sendecka, T. Groothuis, L. Gustafsson, and B. Doligez. 2009. Heritable variation in maternal yolk hormone transfer in a wild bird population. The American Naturalist **174**:557-564.

Tworoger, S., and S. Hankinson. 2006. Collection, processing, and storage of biological samples in epidemiologic studies: sex hormones, carotenoids, inflammatory markers, and proteomics as examples. Cancer Epidemiology, Biomarkers and Prevention **15**:1578-1581.

van Cauter, E., R. Leproult, and D. Kupfer. 1996. Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. Journal of Clinical Endocrinology and Metabolism **81**:2468-2473.

van de Pol, M., and A. Cockburn. 2011. Identifying the critical climatic time window that affects trait expression. The American Naturalist **177**:698-707.

van de Pol, M., and S. Verhulst. 2006. Age-dependent traits: a new statistical model to separate within and between-individual effects. The American Naturalist **167**:766-773.

Van Hout, A., M. Eens, V. Darras, and R. Pinxten. 2010. Acute stress induces a rapid increase of testosterone in a songbird: implications for plasma testosterone sampling. General and Comparative Endocrinology **168**:505-510.

.

Veiga, J., and V. Polo. 2008. Fitness consequences of increased testosterone levels in female spotless starlings. The American Naturalist **172**:42-53.

Verkerk, G., A. Phipps, J. Carragher, L. Matthews, and K. Stelwagen. 1998. Characterisation of milk cortisol concentrations as a measure of short-term stress responses in lactating dairy cows. Animal Welfare **7**:77-86.

Von Engelhardt, N., and T. Groothuis. 2011. Maternal hormones in avian eggs. Pages 91-127 *in* Norris and Lopez, editors. Hormones and Reproduction of Vertebrates 4: Birds. Academic Press.

von Engelhardt, N., P. Kappeler, and M. Heistermann. 2000. Androgen levels and female social dominance in Lemus catta. Proceedings of the Royal Society B: Biological Science **267**:1533-1539.

Vreeburg, J., P. Woutersen, M. Ooms, and J. Tenbosch. 1981. Androgens in the fetal guinea-pig after maternal infusion of radioactive testosterone. Journal of Endocrinology **88**:9-16.

Walker, C., G. Trottier, J. Rochford, and D. Lavallee. 1995. Dissociation between behavioral and hormonal responses to the forces swim stress in lactating rats. Journal of Neuroendocrinology **7**:615-622.

Walker, S., W. Waddell, and K. Goodrowe. 2002. Reproductive endocrine patterns in captive female and males red wolves (Canis rufus) assessed by fecal and serum hormone analysis. Zoo Biology **21**:321-335.

Walling, C., D. Nussey, A. Morris, T. Clutton-Brock, L. Kruuk, and J. Pemberton. 2011. Inbreeding depression in red deer calves. BMC Evolutionary Biology **11**:318.

Walling, C., J. Pemberton, H. J, and L. Kruuk. 2010. Comparing parentage inference software: reanalysis of a red deer pedigree. Molecular Ecology **19**:1914-1928.

Wang, J. 2004. Sibship reconstruction from genetic data with typing errors. Genetics **166**:1963-1979.

Wang, J., and A. Santure. 2009. Parentage and sibship inference from multilocus genotype data under polygamy. Genetics **181**:1579-1594.

Ward, I., and J. Weisz. 1980. Maternal stress alters plasma testosterone in fetal males. Science **207**:328-329.

Ward, I., and J. Weisz. 1984. Differential effects of maternal stress on circulating levels of corticosterone, progesterone and testosterone in male and female rat fetuses and their mothers. Endocrinology **114**:1635-1644.

Ward, O., I. Ward, J. Denning, J. French, and S. Hendricks. 2002. Postparturitional testosterone surge in male offspring of rats stressed and/or fed ethanol during late pregnancy. Hormones and Behavior **41**:229-235.

Watson, R., C. Munro, K. Edwards, V. Norton, J. Brown, and S. Walker. 2013. Development of a versatile enzyme immunoassay for non-invasive assessment of glucocorticoid metabolites in a diversity of taxonomic species. General and Comparative Endocrinology **186**:16-24.

Weingrill, T., D. Gray, L. Barrett, and P. Henzi. 2004. Fecal cortisol levels in free-ranging female chacma baboons: relationship to dominance, reproductive state and environmental factors. Hormones and Behavior **45**:259-269.

While, G., C. Isaksson, J. McEvoy, D. Sinn, J. Komdeur, E. Wapstra, and T. Groothuis. 2010. Repeatable intra-individual variation in plasma testosterone concentration and its sex-specific link to aggression in a social lizard. Hormones and Behavior **58**:208-213.

Whitten, P., D. Brockman, and R. Stavisky. 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. Yearbook of Physical Anthropology **41**:1-23.

Williams, T. 2008. Individual variation in endocrine systems: moving beyond the 'tyranny of the Golden Mean'. Philosophical Transactions of the Royal Society B: Biological Sciences **363**:1687-1698.

Williams, T., C. Ames, and Y. Kiparissis. 2005. Laying-sequence-specific variation in yolk oestrogen levels, and relationship to plasma oestrogen in female zebra finches (Taeniopygia guttata). Proceedings of the Royal Society B: Biological Sciences **272**:173-177.

Wilson, A., A. Charmantier, and J. Hadfield. 2008. Evolutionary genetics of ageing in the wild: empirical patterns and future perspectives. Functional Ecology **22**:431-442.

Wilson, A., D. Reale, M. Clements, M. Morrissey, E. Postma, S. Walling, L. Kruuk, and D. Nussey. 2010. An ecologist's guide to the animal model. Journal of Animal Ecology **79**:13-26.

Wingfield, J. 2003. Control of behavioral strategies for capricious environments. Animal Behaviour **66**:807-816.

Wingfield, J., and D. Farner. 1980. Control of seasonal reproduction in temperate-zone birds. Progress in Reproductive Biology **5**:62-101.

Wingfield, J., R. Hegner, A. Dufty, and G. Ball. 1990. The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems and breeding strategies. American Naturalist **136**:829-846.

Wingfield, J., S. Lynn, and K. Soma. 2001. Avoiding the 'costs' of testosterone: Ecological bases of hormone-behavior interactions. Brain, Behavior and Evolution **57**:239-251.

.

Wingfield, J., and R. Sapolsky. 2003. Reproduction and resistance to stress: when and how. Journal of Neuroendocrinology **15**:711-724.

Wingfield, J., and B. Silverin. 1986. Effects of corticosterone on territorial behavior of free-living male song sparrows Melospiza melodia. Hormones and Behavior **20**:405-417.

Wolf, J., and M. Wade. 2009. What are maternal effects (and what are they not)? Philosophical Transactions of the Royal Society B: Biological Sciences **264**:1107-1115.

Young, E., J. Abelson, and S. Lightman. 2004. Cortisol pulsatility and its role in stress regulation and health. Frontiers in Neuroendocrinology **25**:69-76.

Zuk, M., T. Johnsen, and T. Maclarty. 1995. Endocrine-immune interactions, ornaments and mate choice in red jungle fowl. Proceedings of the Royal Society of London B: Biological Sciences **260**:205-210.

Zumoff, B., R. Rosenfeld, G. Strain, J. Levin, and D. Fukushima. 1980. Sex differences in the twenty-four-hour mean plasma concentrations of dehydroisoandrosterone (DHA) and dehydroisoandrosterone sulfate (DHAS)

and the DHA and DHAS ratio in normal adults. Journal of Clinical Endocrinology and Metabolism **51**:330-333.

Zumoff, B., G. Strain, L. Miller, and W. Rosner. 1995. Twenty-four-hour mean plasma testosterone concentration declines with age in normal premenopausal women. Journal of Clinical Endocrinology and Metabolism **80**:1429-1430.

Zysling, D., T. Greives, C. Breuner, J. Casto, G. Dema, and E. Ketterson. 2006. Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (Junco hyemalis carolinensis). Hormones and Behavior **50**:200-207.

Appendices

APPENDIX A | Handling stress

Changes in testosterone and cortisol levels with handling

A.1. Introduction

Blood sampling is an invasive procedure which is usually preceded by a period of catching and/or handling of the target animal (though see remote devices such as the "DracPac": Ingram et al. 1999). This can make the sampling of baseline hormone levels problematic because of the potential for neuroendocrine responses to be initiated by that interaction. This is particularly problematic in wild animals which will not be habituated to capture and handling, and so are likely to perceive it as a threat.

The rapid neuroendocrine response to acute stressors is characterised by an increase in glucocorticoids (Moberg 2000, Romero 2004, Romero and Butler 2007, Table A1.1). There is also evidence that circulating levels of other hormones, including testosterone, respond rapidly to stressors (Armario and Castellanos 1984, Moore et al. 1991, Deviche et al. 2010, Van Hout et al. 2010, Deviche et al. 2012), however the direction of the testosterone response is less consistent between studies. Whilst glucocorticoid levels always increase in response to stressors (Romero and Butler 2007) testosterone may either increase (Armario and Castellanos 1984, Van Hout et al. 2010) or decrease (Cumming et al. 1983, Deviche et al. 2010, Deviche et al. 2012). Research in primates suggests that the direction of this testosterone response also varies between individuals within the same population depending on their social status (Sapolsky 1986). Ultimately, however, even when testosterone levels do change under acute stress, this response appears slower than that of glucocorticoids (e.g. Moore et al. 1991). Studies in birds, for example, show testosterone to respond after around 10 minutes post-stressor (Van Hout et al. 2010, Deviche et al. 2012), whilst glucocorticoids can increase within 3 minutes of handling (Romero and Reed 2005). The particularly rapid nature of the glucocorticoid response means that obtaining measures of circulating "baseline" concentrations can be problematic

when individuals require catching before blood samples can be collected (e.g. in wild populations).

Whilst the effects of acute stress on circulating hormone levels is less studied in ungulates than in birds, domestic cattle do show pronounced and rapid increases in cortisol levels following manipulation such as dehorning or manual restraint (McMeekan et al. 1997, Graf and Senn 1999). Many of these ungulate studies, however, tend to have a low sampling frequency, with the first sample not collected until at least 15-20 minutes after the stressor (see Table A. 1). This often means that cortisol levels have already peaked before the first samples are collected (though see Graf and Senn 1999), with no indication as to how soon after the stressor cortisol levels begin increasing from baseline.

As part of ongoing research on the Isle of Rum NNR, red deer calves are caught soon after birth in order to collect morphometric data and biological samples including blood (discussed in chapter 2). Traditionally, the calves are bled after around 4-5 minutes of restraint; however it remained unknown as to whether hormone concentrations in these blood samples were representative of a calf’s baseline circulating levels, or whether they were confounded by handling stress. In order to address this knowledge gap, this study tested the responsiveness of circulating testosterone and cortisol levels to the traditional sampling methodology by collecting blood samples at two time points during calf restraint.

Table A. 1: *Time from initiation of stressor to glucocorticoid (GC) peak for domestic and wild ungulates.*

Species	Stressor	Time to GC peak	Sample freq (min)	Reference
Cattle (4-6 w.o.)	Dehorning & simulated dehorning	10-20 min [a]	At 5,10,20,40,60,90	Graf & Senn (1999)
Cattle (15 w.o. ♀)	Restraint & horn scoop vs control	≤15 min	15	McMeekan et al. (1997)
Cattle (~6 m.o.)	Dehorning & head restraint controls	≥10-30 min [a]		Cooper et al. (1995)
Pig (3-12 w.o.)	Castration & control	≤30 min	30	Carroll et al. (2006)
Sheep (5, 21, 42 d.o.)	Tail docking & castration	30-40 min [a]	12	Kent et al. (1993)
Chamois	Drive net capture	≤60 min	60	Lopez-Olivera et al. (2007)
Red deer (♀)	Handling & herding	≤30 min	30	Diverio et al. (1996)
Roe deer	Drive net capture	≤60 min	60	Montane et al. (2003)
Vicuna	ACTH challenge	60 min	30	Bonacic et al. (2003)

[a] depending on treatment

A.2. Methods

Concentrations of testosterone were measured in 36 blood samples (from 18 individuals) and concentrations of cortisol were measured in 42 blood samples (from 21 individuals) collected between 26th May and 5th June 2013 from red deer calves born on the Isle of Rum NNR in 2013. Two blood samples were collected from each calf: one immediately after being caught, and the other 4-5 minutes later when blood samples are conventionally collected as part of the ongoing study (see chapter 2.1.1 for full details of blood collection and processing).

Hormones were extracted, and testosterone concentrations measured, following previous methodology (see chapter 2.2 & 2.3 for full details). The intra-assay CV for the testosterone assay was calculated at 4.66% (no inter-assay CV exists because all samples were run on a single assay plate).

In addition to testosterone, cortisol concentrations were measured (in ng/ml) using a commercially available cortisol competitive-binding EIA kit (500360, Cayman Chemicals Ltd., USA), with all samples run in duplicate. The cross-reactivity was given as: cortisol 100%, prednisolone 4%, cortexolone 1.6%, and <1% for all remaining compounds tested including corticosterone (0.14%), cortisone (0.13%), androstenedione, estrone and testosterone (all <0.01%). Serial dilutions of pooled samples showed high parallelism with the standard curve ($p<0.001$) and no samples fell below the calculated LOD (0.076 ng/ml). The intra- and inter-assay CVs were calculated at 9.98% and 10.69% respectively.

Both testosterone and cortisol concentrations were log-transformed to normalise the residuals prior to analysis, and paired t-tests were used to explore any direct relationship between an individual's hormone concentrations at first and second bleed.

A.3. Results

After log-transforming the hormone concentrations to normalise the residuals, there was an overall mean decrease in testosterone levels (Table A. 2; Figure A. 1), however a paired t-test did not find this difference to be significant (paired t-test: t=0.112, df=17, p=0.912). In contrast, there was a significant mean increase in circulating cortisol levels between the two time periods (paired t-test: t=2.497, df=20, p=0.021; Table A. 2; Figure A. 1). This indicates that circulating cortisol, but not testosterone, levels increased over the 5 minute period of handling between the two sampling periods.

Table A. 2: *Mean circulating testosterone and cortisol levels from calves when they are first captured ("first bleed") and then approx. 5 minutes later ("second bleed"). The mean difference between first and second bleeds is also given. SE estimates are given in parentheses.*

	First bleed	Second bleed	Mean diff
Testosterone (ng/ml)	1.376 (0.384)	1.011 (0.229)	-0.401 (0.318)
Cortisol (ng/ml)	14.277 (3.469)	19.515 (2.986)	5.238 (3.884)

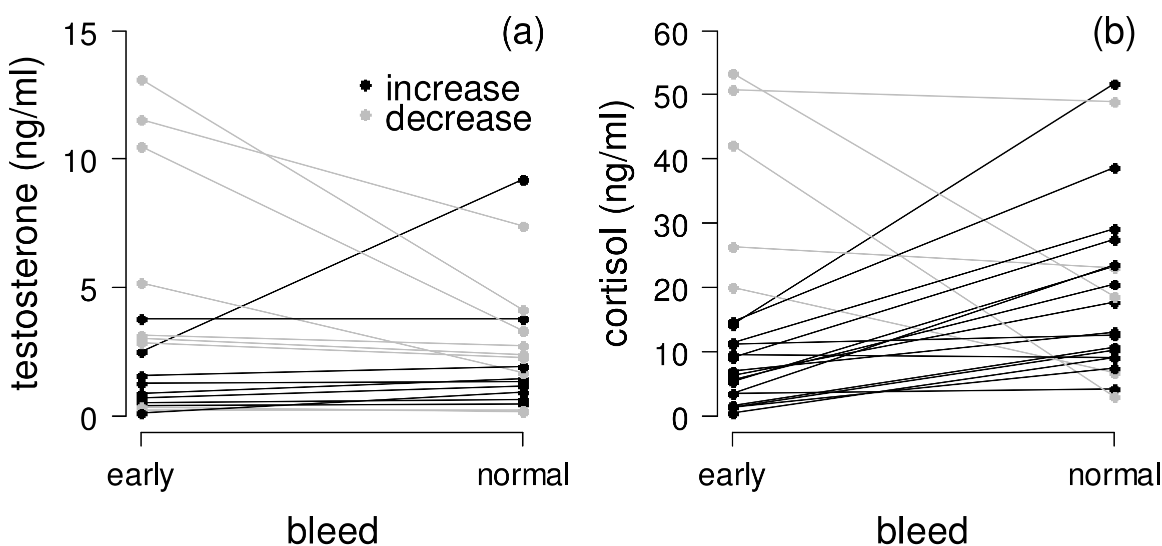


Figure A. 1: *Change in circulating levels of (a) testosterone and (b) cortisol from first ("early") and second ("normal") bleeds.*

A.4. Discussion

As has been reported previously (chapters 3 & 4), circulating testosterone levels varied considerably between red deer calves in this population. Overall, however, there was no consistent change in testosterone concentration in the 4-5 minutes between first and second bleeds. This indicates that circulating levels in these red deer calves did not respond rapidly, or consistently, to perceived handling stress, and concurs with previous bird studies which do not find significant changes in testosterone until around 10 minutes after a stressor (Deviche et al. 2012). There was also considerable variation in the direction and magnitude of change in measured testosterone levels, which previous studies in adult primates attribute to variance in social status (Sapolsky 1986). Due to the very young age of the study calves (<2 weeks old), however, the individual differences seen in this population are more likely to be a consequence of stochastic variation in circulating concentrations. Testosterone secretion is pulsatile in red deer, and naturally fluctuates over short periods of time (Suttie et al. 1992).

In contrast to testosterone, circulating cortisol levels did increase over the 4-5 minutes of processing between first and second bleeds. This concurs with previous studies in birds which show that circulating glucocorticoids can begin increasing with 2-3 minutes of experiencing a stressor (Romero and Reed 2005), and confirms that capture and handling are perceived as stressful events to the red deer calves. The degree of change, however, was not consistent among individuals, and of the 21 calves sampled, 5 showed a *decrease* in cortisol concentration between the two time periods. Given that little is known about individual calf experiences prior to capture, this may reflect individuals recovering from previous unknown stressors, or may be a consequence of variation in stress perception (Greenberg et al. 2002). As with circulating testosterone, cortisol secretion is also pulsatile (Ingram et al. 1999), and may account for variation arising between individuals.

Overall, these trial analyses showed that circulating testosterone concentrations did not change consistently from capture (first bleed) to the time of regular blood collection around 5 minutes later (second bleed). From this subset of data, it can

therefore be concluded that handling effects are unlikely to confound the results presented in chapters 3 & 4. Circulating levels of cortisol, however, did show an overall increase between the two time points, as well as considerably inter-individual variation in the degree of their response. This indicates that blood samples collected at the second time period cannot be considered representative of baseline cortisol levels, and are confounded by catching and handling, as well as individual differences in their responses to these as stressors. As a consequence, individual variation in cortisol levels in the archived blood samples was not explored any further.

APPENDIX B| Neonatal testosterone & weather

Effect of weather during foetal development on a calf's circulating testosterone levels

B.1. Introduction

After accounting for the role of maternal and calf traits (chapter 3), as well as heritability (chapter 4), in explaining a calf's circulating testosterone levels, a statistically significant component continued to be explained by variation across years. It was therefore speculated that a calf's circulating concentrations may also be affected by climatic conditions during foetal development. Testosterone is primarily secreted from the gonads in both males and females, which begin developing and differentiating early in development (Lincoln 1973).

Environmental effects during gestation are likely to act on the developing foetus indirectly via variation in the maternal environment. Extreme heat stress, for example, retards mammalian foetal development in experimental systems (Hansen 2009), although whether these effects translate into natural systems where temperature fluctuations are less extreme is unknown. The indirect effects of the mother might also be mediated by variation in maternal condition due to energetic demands or food availability (Albon et al. 1987, Robinson et al. 1999). Such effects may have consequences for foetal development due to variation in maternal, and thus foetal, stress. Exposure to prenatal stress has been shown to have long-term effects on an individual's maturation, behaviour and endocrinology (Ward and Weisz 1984, Seckl 2004, Kaiser and Sachser 2005). Within this red deer study population, however, there was no evidence of a link between an endocrine measure of maternal stress (FCM levels) during pregnancy and offspring testosterone levels (chapter 6). Furthermore, maternal FCM levels did not show any among-year variation (chapter 6), so differences in environmental conditions between years were not sufficient to translate into clear annual differences in

maternal stress hormone levels. Whilst this suggests that the cohort effects observed in chapters 3 & 4 were not a consequence of foetal exposure to maternal glucocorticoids, among-cohort variation might still arise as a consequence of population-level effects on maternal condition.

Female condition will change over time as resource availability and access changes, much of which will be heavily influenced by local weather conditions. For example, low temperatures (combined with high winds and rain) will put higher metabolic demands on the body, and result in greater heat loss. This can also result in behavioural changes, with females less likely to graze on the high quality but exposed *Agrostis/Festuca* grassland during poor weather because of the excessive energy costs of maintaining body temperature in such an exposed habitat (Conradt et al. 2000). Consequently, during periods of cold, wet and windy weather, females are likely to have less excess energy to invest in foetal development. Annual variation in food quality and availability will also influence the amount of energy intake. For red deer on the Isle of Rum, the quality of grazing is heavily dependent on weather conditions. For example, the productivity of *Agrostis/Festuca* grassland is lower when the growing season is dominated by cooler weather (Langvatn et al. 1996), although Langvatn et al. (1996) suggests that this slower growth might lead to higher *quality* graze. When conditions are considered "bad" (e.g. cold, windy, wet winter-spring: Conradt et al. 2000), females are more vulnerable to heat loss and malnutrition. If females in poorer condition do have calves with less testosterone (as proposed in chapter 3), then calves born after critical periods of high wind, low temperature or high rainfall might be expected to have lower circulating concentrations.

Variation in the maternal environment would not be expected to affect the foetus consistently across gestation, but rather to have the largest impact during key time periods of development. In rats, for example, maternal glucocorticoid levels tend to have the largest effect on offspring birth weight if experienced towards the *end* of gestation when foetuses are growing the fastest (Nyirenda et al. 1998). Give than gonadal development in red deer occurs early in foetal life (Lincoln 1973), environmental conditions during the first few months of gestation are likely to be

particularly critical in determining the circulating testosterone levels of the resulting calf.

The aims of this preliminary analysis were to (a) identify critical time windows during gestation when weather variables explained the most variation in circulating calf testosterone levels, and (b) explore the effects of varying weather conditions during these time periods.

B.2. Methods

This study investigated the potential for neonatal testosterone levels to be affected by weather conditions during foetal development, making use of circulating testosterone concentrations measured and explored in calves in chapters 3 & 4 (n=854 samples). Full methods for sample collection, hormone extraction, and testosterone assaying are outlined in chapter 2. Because foetal development varies between the sexes (Lincoln 1973), the effects of weather were explored independently in the two sexes.

B.2.1. Weather data

Three weather variables were tested here: daily rainfall (mm), daily mean wind speed (m/s) and daily maximum temperature (°C). These weather measures were recorded on the Isle of Rum NNR by SNH and are available from the Met Office British Atmospheric Data Centre.

Mean values for each sex were calculated for time windows 2-8 weeks in length (in 1 week increments) which were tested across the entire period of foetal development. These time windows were allowed to overlap and spanned the time period starting at the estimated date of conception (235 days prior to birth: Clements et al. 2011) and ending at the date of birth.

B.2.2. Time window identification & testing

The effects of rain, wind, and max temp on circulating calf testosterone levels were tested one-by-one for each sex. The optimal time window was identified for each weather effect by comparing linear mixed effects models (package: lme4, Bates et al. 2011) which included a mean weather measure for each time window in turn. These models also corrected for calf age, sex of previous calf and collection time as fixed effects (see chapter 3.4.1 for description), and maternal identity, year of birth and assay date as random effects. All of these additional effects have previously been shown to significantly influence a calf's testosterone levels (chapter 3). As in the models presented in chapter 3, testosterone was log-transformed to normalise the residuals, and all continuous explanatory variables were centred prior to fitting the models.

Models were ranked by AIC (Akaike information criterion) values and, within each sex, the model which best fit the data (i.e. had the lowest AIC value) was selected for each weather effect. This gave a sex-specific optimal time window for each of the three weather variables. The sex-specific mean values for rain, wind and max temp within these time windows were estimated for each cohort. These were fitted in sex-specific linear mixed-effects models (package: lme4, Bates et al. 2011) along with the fixed and random effects used in testing. The statistical significance of mean rain, mean wind and mean max temp was then explored using maximum likelihood estimates obtained from ANOVAs between nested models with and without the weather variable. Effects were considered statistically significant at $p<0.05$ when dropped from the model. As with the models used in testing, testosterone was log-transformed to normalise the residuals, and all continuous explanatory variables were centred prior to fitting the models.

B.3. Results

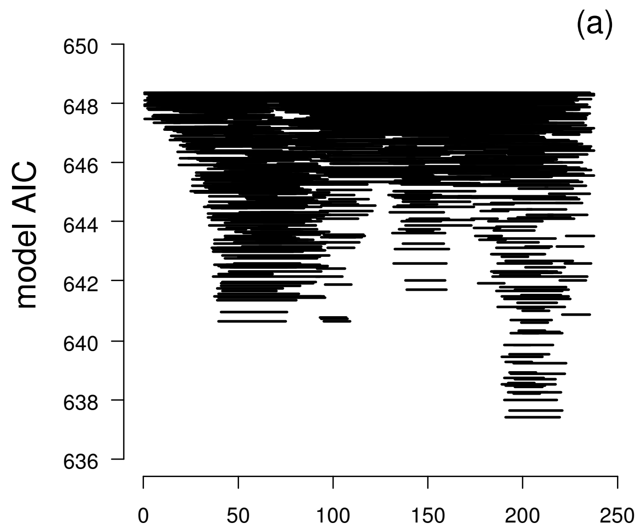
B.3.1. Optimal time window

The optimal time windows were not consistent between weather variables or between sexes. Female testosterone levels were best explained by mean rain during

late spring, by mean wind during winter, and by mean max temp during late autumn (Table B. 1; Figure B. 1). In contrast male testosterone levels were best explained by mean rain and wind in winter around mid-gestation, and by mean max temp during late autumn in the months following the rut (Table B. 1; Figure B. 1).

Table B. 1*: Time windows (measured as days into foetal development) when each weather variable accounted for the most variation observed in neonatal calf testosterone levels. Time windows were identified from mixed effect models correcting for calf and maternal effects known to influence a calf's circulating testosterone concentrations.*

WEATHER	(a) Female calves	(b) Male calves
Mean daily rainfall	191-219	95-144
Mean daily wind speed	99-155	111-167
Mean daily max temp	76-90	45-66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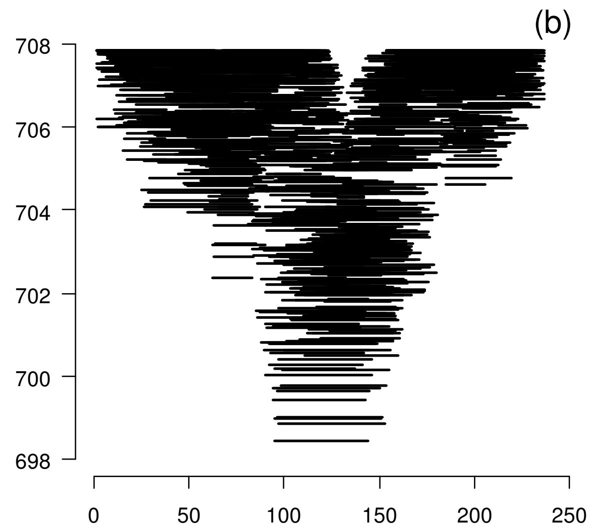
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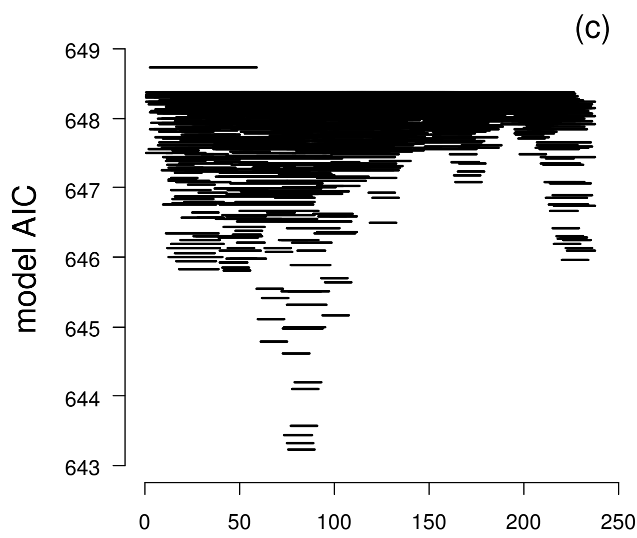
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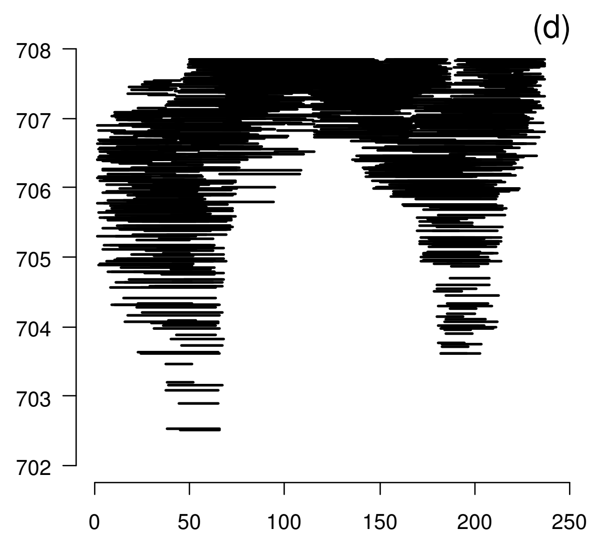
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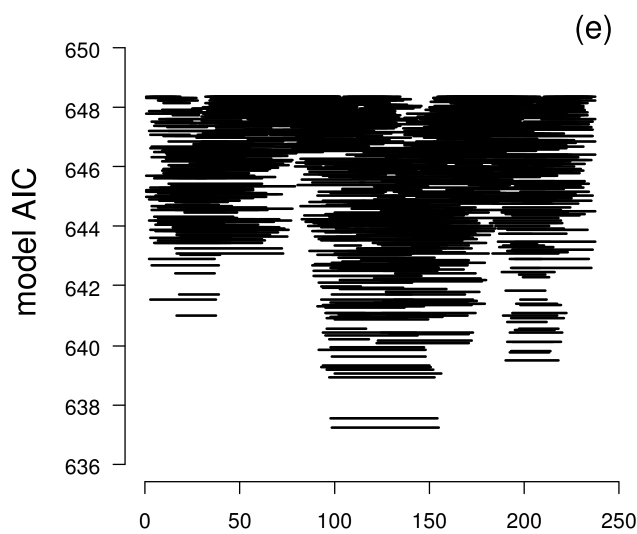
(e)
model AIC
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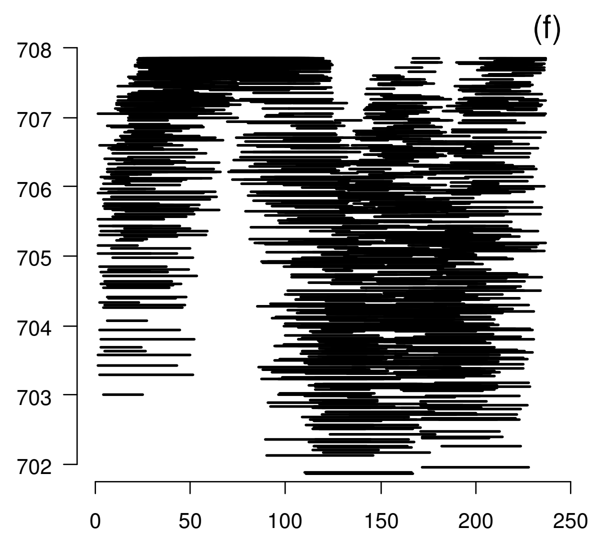
foetal age (days)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foetal age (days)

Figure B. 1: *Comparison of AIC values for models testing the effects of rain (a & b), wind (c & d) and max temp (e & f) on circulating neonatal testosterone levels across different time windows. Possible time windows ranged between 2 and 8 weeks long (in increments of 1 week). The lowest AIC values indicate models which best fit the data. Models were fitted independently for females (a, c & e) and males (b, d & f).*

B.3.2. Weather effects

There was a positive relationship between neonatal testosterone concentration and mean rain during the optimal foetal time window in both females (191-219; p=0.001; Table B. 2; Figure B. 2a) and males (95-144; p<0.001; Table B. 2; Figure B. 2b). There was also a significant positive relationship between mean wind and circulating testosterone levels in females only (99-155; p=0.004; Table B. 2; Figure B. 2c). There was no effect of mean max temp on testosterone levels in calves of either sex (Table B. 2; Figure B. 2e & f).

Table B. 2: *Linear mixed models examining the main effects of weather variables (along with calf and maternal traits) on inter-individual variation in (log-transformed) testosterone amongst (a) female and (b) male calves.*

FIXED EFFECTS	**(a) Female calves (394)**			**(b) Male calves (376)**		
	Est. ± SE	*p*		Est. ± SE	*p*	
(Intercept)	4.365 ± 0.174	<0.001	***	4.078 ± 0.233	<0.001	***
After 24 hrs	0.863 ± 0.165	<0.001	***	1.175 ± 0.205	<0.001	***
Sex of previous calf (♂)	-0.082 ± 0.055	0.145		-0.138 ± 0.061	0.035	*
Collection time	-0.017 ± 0.010	0.095	.	-0.038 ± 0.010	<0.001	***
Calf age: <24 hours	-0.044 ± 0.004	<0.001	***	-0.053 ± 0.005	<0.001	***
Calf age: >24 hours	-0.002 ± 0.001			0.001 ± 0.001		
Mean daily rainfall	0.048 ± 0.017	0.001	**	0.038 ± 0.018	<0.001	***
Mean daily wind speed	0.106 ± 0.032	0.004	**	0.054 ± 0.049	0.290	
Mean daily max temp	-0.023 ± 0.021	0.292		0.042 ± 0.031	0.120	
RANDOM EFFECTS	**Var.**	***p***		**Var.**	***p***	
Mother ID (n=227/219)	0.014	0.432		0	1	
Birth year (n=15)	0.002	0.825		0.015	0.090	.
Date of assay (n=7)	0.035	0.004	**	0.093	0.001	**
Residual	0.248			0.326		

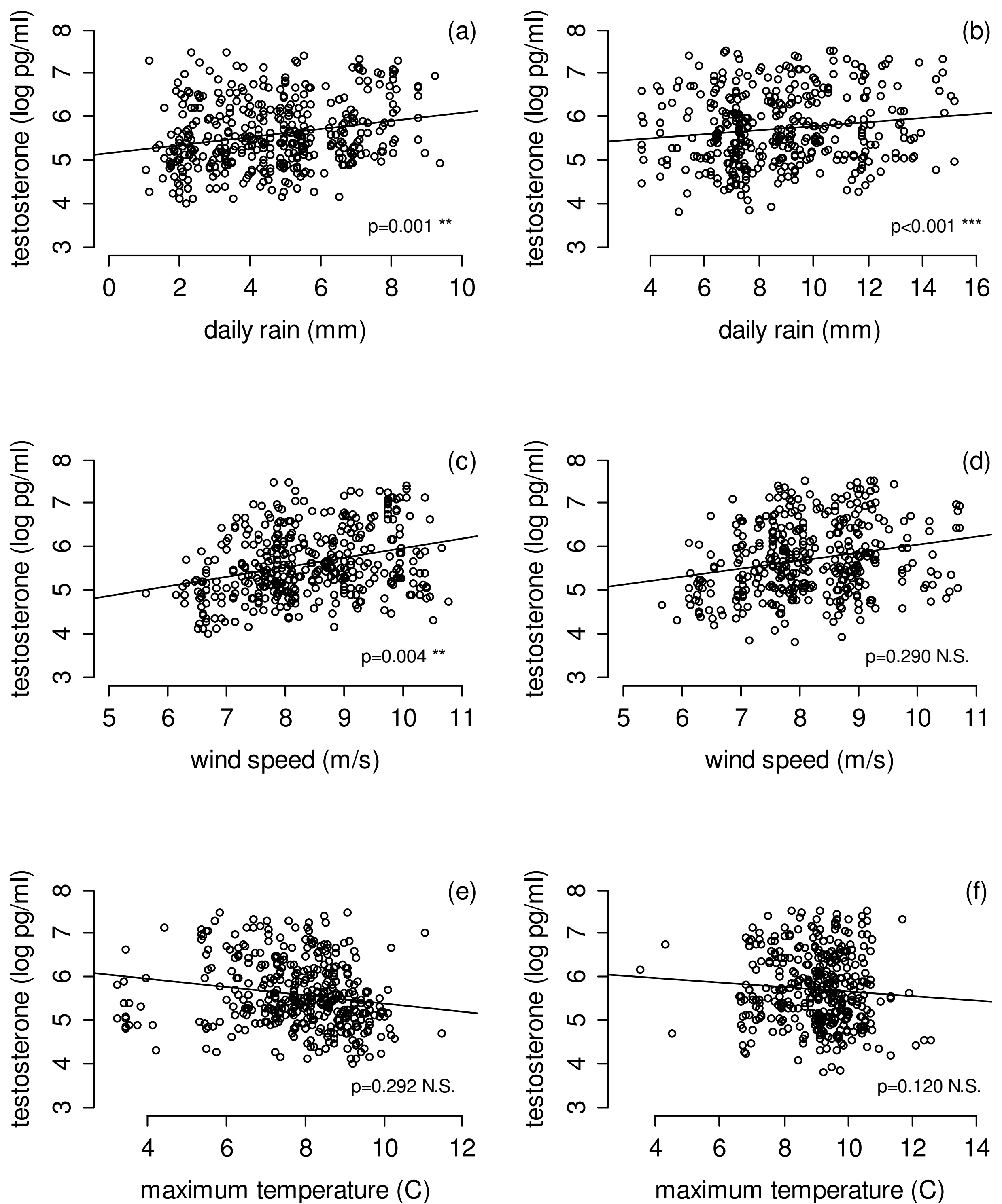

Figure B. 2: *Variation in log-transformed neonatal testosterone with daily rainfall (a & b), mean wind speed (c & d), and maximum temperature (e & f). The figures show the raw data, and the smooth lines were fitted from regressions of log-transformed hormone concentrations against the specific weather variable. Models were fitted independently for females (a, c & e) and males (b, d & f).*

B.4. Discussion

Results from these preliminary analyses indicated that weather conditions during foetal development could be important in influencing the circulating testosterone levels of the resulting calf. Whilst evidence suggests that a neonate's hormone levels are endogenously produced rather than being a product of the mother (Vreeburg et al. 1981, Tapanainen 1983, Despres et al. 1984), maternal condition during key periods of pregnancy may still be important in influencing foetal development. This is known to be the case for other traits such as birth weight (Albon et al. 1987).

These preliminary analyses did not identify any one period of time when weather conditions were particularly important. Female calf testosterone levels were most closely linked to rainfall in the penultimate month before birth, and to wind speed during the middle of gestation. Male testosterone levels were also most closely associated with rainfall during mid-gestation, but not with wind speed. Neither female nor male testosterone concentrations were associated with maximum temperature. All the significant time periods fall after the initial stages of rapid gonadal growth in red deer (45-95 days into gestation: Lincoln 1973), indicating that this relationship was not driven by direct effects on early gonadal development.

Where climatic conditions did significantly relate to offspring testosterone levels there was one consistency: higher levels of testosterone were found in individuals which developed when the mother was exposed to higher rainfall or wind speed during the critical time periods. Wet and windy winter/spring periods are generally considered "bad" conditions for red deer on Rum (Coulson et al. 1998, Conradt et al. 2000) because they are associated with a high energetic cost. This runs contrary to the expectation that bad weather would be associated with low testosterone levels due to poor condition mothers investing less in their offspring's development. Wet and windy winters are, however, associated with higher levels of failed conceptions and foetal abortion/reabsorption in gestating females (Albon et al. 1986). A positive relationship between maternal condition and offspring testosterone many, therefore, still remain if females in poor condition are more likely to abort or reabsorb their developing

foetuses. As a consequence, females entering gestation in better condition may be more likely to carry their foetus to full term during years when weather conditions are harsh. If the condition of the mother is to be more explicitly tested, then it would be important for future analyses to expand the range of time windows to encompass the summer months prior to the rut. Weather might play an important role during the summer months by influencing plant productivity and thus the resources available to the grazing deer (Langvatn et al. 1996, Mysterud et al. 2008). Conditions during this period of time may therefore determine both the condition of the mother when she enters the rutting season and the amount of resources stored for the winter period.

These analyses utilised a sliding window modelling approach which is computationally simple and relies on identifying the aggregate time period that best correlates with the trait of interest. The simplicity of this model does, however, have disadvantages in that the time windows have arbitrary cut-off points which are not biologically meaningful, and rely on the assumption that each day within that time window is equally important (van de Pol and Cockburn 2011). In this analysis, the time windows were also calculated independently for each weather variable. In order to obtain more accurate time window estimates, all other weather variables should be included as fixed effects in the models. Whilst these analyses are only preliminary, the results clearly indicate that weather conditions during foetal development are an important factor to consider when exploring neonatal hormone levels.

APPENDIX C| Heritability of faecal hormone concentrations

In appendix C I estimate the heritability of FAM and FCM concentrations in both females and males. Models were fitted to the data in ASReml-R 3.0.3 (Butler 2009) for each of the four sex-hormone combinations. Each model included the fixed effects previously explored in the respective female (chapter 6) and male (chapter 5) chapters. The total phenotypic variance (V_P) was then partitioned into additive genetic variance (V_A), which was estimated using relatedness information in the pedigree (based on maternal and paternal identification from a combination of behavioural and genetic data: Walling et al. 2010), individual identity (V_{IDE}; to account for repeat sampling), and the unexplained residual variance (V_R). The significances of the three variance components (V_A, V_{IDE}, and V_R) were tested using LRTs, comparing models with and without the relevant random effect (see chapter 4.4.1 for more details of the method). The heritability of each was then estimated as the proportion of V_P that was accounted for by V_A.

There was no evidence of faecal hormone concentrations being heritable in either sex after removing the effects of individual identity (V_{IDE}) which corrects for the repeated sampling of individuals (Table C. 1). There is, therefore, no scope for either FAM or FCM concentrations to evolve in this population, even if appropriate selection pressures exist.

Table C. 1: *Components of variance in FAM (a & c) and FCM (b & d) levels collected from female (a & b) and male (c & d) red deer. Sample sizes are in parentheses.*

(a) Female FAM (638)

	Component ± SE	*p*	Proportion of var. ± SE
V_A	0.015 ± 0.019	0.505	0.023 ± 0.027
V_{IDE}	0 ± NA	1	0
V_R	0.657 ± 0.041	<0.001	0.977 ± 0.027

(b) Female FCM (768)

	Component ± SE	*p*	Proportion of var. ± SE
V_A	0.009 ± 0.013	0.367	0.014 ± 0.020
V_{IDE}	0 ± NA	1	0
V_R	0.633 ± 0.035	<0.001	0.986 ± 0.020

(c) Male FAM (141)

	Component ± SE	*p*	Proportion of var. ± SE
V_A	0.322 ± 0.753	0.595	0.130 ± 0.301
V_{IDE}	0.504 ± 0.776	0.451	0.203 ± 0.309
V_R	1.652 ± 0.282	<0.001	0.667 ± 0.128

(d) Male FCM (178)

	Component ± SE	*p*	Proportion of var. ± SE
V_A	0 ± NA	1	0
V_{IDE}	0.105 ± 0.056	0.054	0.189 ± 0.090
V_R	0.447 ± 0.057	<0.001	0.810 ± 0.090

APPENDIX D| Chapter 4 supporting material

Table D. 1: *Fixed effects for univariate animal model explaining variance in calf neonatal testosterone concentrations. See Table 4.2 for variance component estimates.*

<table>
<tr><th>FIXED EFFECTS</th><th>Est. ± SE</th><th>p</th></tr>
<tr><td>Calf age</td><td>-1.252 ± 0.073</td><td><0.001 ***</td></tr>
<tr><td>Calf age <24 hours</td><td>-0.046 ± 0.003</td><td rowspan="2"><0.001 ***</td></tr>
<tr><td>Calf age >24 hours</td><td>-0.001 ± 0.001</td></tr>
<tr><td>Calf sex</td><td>0.122 ± 0.037</td><td>0.001 **</td></tr>
<tr><td>Sex of previous calf</td><td>-0.092 ± 0.038</td><td>0.017 *</td></tr>
<tr><td>Collection time</td><td>-0.024 ± 0.007</td><td><0.001 ***</td></tr>
<tr><td>Assay date</td><td>7 factor levels</td><td><0.001 ***</td></tr>
</table>

APPENDIX E| Chapter 5 supporting material

Table E. 1: *Multivariate mixed effects model estimating the main effects of extrinsic factors on individual-level variation in (a) FAM concentrations, (b) FCM concentrations and (c) reproductive effort when FAM measures falling below the LOD (n=34) were included in the analyses. These <LOD samples were assigned the LOD value (0.89 ng/ g faeces) because this was the lowest measure that could be reliably estimated from the standard curve.*

FIXED EFFECTS	FAM (175)			FCM (178)			Reproductive effort (2833)		
	Est. ± SE	*p*		Est. ± SE	*p*		Est. ± SE	*p*	
Age	-0.032 ± 0.073	0.146		0.057 ± 0.031	0.019	*	0.174 ± 0.009	<0.001	***
Age^2	-0.027 ± 0.012	<0.001	***	-	-		-0.038 ± 0.002	<0.001	***
February [a]	-0.053 ± 0.898			0.367 ± 0.316			-		
March [a]	-0.763 ± 1.006			0.364 ± 0.326			-		
April [a]	-0.108 ± 0.872			0.037 ± 0.307			-		
May [a]	-0.029 ± 1.459			0.186 ± 0.419			-		
June [a]	-0.834 ± 1.920	0.047	*	-0.119 ± 0.678	<0.001	***	-	-	
July [a]	0.091 ± 1.038			-0.096 ± 0.344			-		
August [a]	0.380 ± 0.731			0.783 ± 0.254			-		
September [a]	0.923 ± 0.693			0.790 ± 0.248			-		
October [a]	1.009 ± 0.748			0.783 ± 0.258			-		
November [a]	-0.255 ± 0.891			0.453 ± 0.313			-		
Age at final sample	0.049 ± 0.068	0.527		-0.005 ± 0.032	0.870		-	-	
Assay date	*7 estimates*	<0.001	***	*7 estimates*	<0.001	***	-	-	

[a] Estimates for month are relative to estimates of January

***Table E. 2**: Multivariate mixed effects model estimating variances (diagonal), correlations covariances (below diagonal), and (above diagonal) for FAM, FCM and reproductive effort at (a) among-individual and (b) residual within-individual levels (SE in parentheses) when FAM measures falling below the LOD (n=34) were included in the analyses. Shaded cells indicate values that were fixed and not allowed to vary. Statistically significant variances and covariances are in bold.*

(a) among-individual

	FAM	FCM	Effort
FAM	0 (NA)	0	0
FCM	0	**0.175** **(0.067)** $\boldsymbol{\chi^2=3.859}$ ***p=0.005***	0.591 (0.181)
Effort	0	**0.205** **(0.077)** $\boldsymbol{\chi^2=3.349}$ ***p=0.010***	**0.684** **(0.068)** $\boldsymbol{\chi^2=110.294}$ ***p<0.001***

(b) within-individual

	FAM	FCM	Effort
FAM	**2.995** **(0.345)** $\boldsymbol{\chi^2=181.446}$ ***p<0.001***	-0.212 (0.101)	0.017 (0.118)
FCM	-0.221 (0.111) $\chi^2=1.588$ *p=0.204*	**0.365** **(0.050)** $\boldsymbol{\chi^2=64.501}$ ***p<0.001***	0.336 (0.126)
Effort	0.034 (0.234) $\chi^2=0.011$ *p=0.884*	**0.235** **(0.090)** $\boldsymbol{\chi^2=2.678}$ ***p=0.021***	**1.311** **(0.041)** $\boldsymbol{\chi^2=9574.2}$ ***p<0.001***

Table E. 3: *Multivariate mixed effects model estimating variances for FAM, FCM and reproductive effort at (a) among-individual, (b) among-year and (c) residual within-individual levels (SE in parentheses). Statistically significant variances are in bold.*

(a) among-individual

	FAM	FCM	Effort
FAM	0.028 (0.148) $X^2=0.095$ p=0.664		
FCM		**0.142 (0.061) $X^2=5.761$ p<0.001**	
Effort			**0.686 (0.061) $X^2=110.848$ p<0.001**

(b) among-year

	FAM	FCM	Effort
FAM	<0.001 (NA) $X^2=0.002$ p=0.945		
FCM		<0.001 (NA) $X^2=0.064$ p=0.720	
Effort			<0.001 (<0.001) $X^2=0.321$ p=0.492

(c) within-individual

	FAM	FCM	Effort
FAM	**1.492 (0.231) $X^2=253.890$ p<0.001**		
FCM		**0.355 (0.049) $X^2=61.229$ p<0.001**	
Effort			**1.311 (0.041) $X^2=9574.218$ p<0.001**

APPENDIX F| Chapter 6 supporting material

Table F. 1: *Fixed effects from multivariate mixed model examining the main effects of season and state on individual variation in female (a) FAM and (b) FCM concentrations when measures below the LOD (FAM: n=39; FCM: n=24) were included in the analyses. These <LOD samples were assigned the LOD value (FAM: 0.89 ng/ g faeces; FCM: 3.51 ng/g faeces) because these were the lowest measures that could be reliably estimated from the standard curve.*

FIXED EFFECTS	(a) FAM (677)			(b) FCM (789)		
	Est. ± SE	*p*		Est. ± SE	*p*	
Age	-0.002 ± 0.020	0.220		0.043 ± 0.018	<0.001	***
Age at final sample	-0.003 ± 0.020	0.883		-0.032 ± 0.018	0.087	.
February [a]	-0.096 ± 0.214			0.023 ± 0.203		
March [a]	0.053 ± 0.225			0.204 ± 0.215		
April [a]	-0.084 ± 0.240			0.439 ± 0.230		
May [a]	0.293 ± 0.303			0.406 ± 0.294		
June [a]	0.237 ± 0.281			0.829 ± 0.277		
July [a]	0.237 ± 0.390	<0.001	***	0.826 ± 0.360	<0.001	***
August [a]	-0.060 ± 0.202			0.307 ± 0.187		
September [a]	-0.038 ± 0.192			0.523 ± 0.181		
October [a]	0.013 ± 0.225			0.386 ± 0.217		
November [a]	0.018 ± 0.253			0.161 ± 0.240		
December [a]	-0.327 ± 0.896			1.094 ± 0.901		
Pregnant (late) [b]	0.277 ± 0.124	0.021	*	0.109 ± 0.123	0.949	
Lactating (♀ calf) [c]	-0.036 ± 0.084	0.936		0.036 ± 0.084	0.022	*
Lactating (♂ calf) [c]	-0.006 ± 0.094			0.242 ± 0.088		
Assay date	*8 factor levels*	<0.001	***	*8 factor levels*	<0.001	***
Time to freezing	0.001 ± <0.001	0.007	**	<0.001 ± <0.001	0.412	
Age:Pregnant (late) [b]	-	-		0.060 ± 0.024	0.009	**

[a] monthly variance estimates are relative to January estimates

[b] pregnancy status relative to non- & early- pregnant females

[c] lactation status relative to non-lactating females

Table F. 2: *Estimates of variance (diagonal), covariance (below diagonal) and correlation (above diagonal) components from the multivariate mixed effects models with hormone measures falling below the LOD included in the analyses (see Table F. 1 for fixed effects).*

(1)EARLY

(a) Among-individual

	FAM	FCM	Calf T
FAM	0.002 (0.020) $p=0.944$ $X^2=0.002$		
FCM		0.014 (0.019) $p=0.508$ $X^2=0.219$	
Calf T			

(b) Among-year

	FAM	FCM	Calf T
FAM	0.001 (0.002) $p=0.987$ $X^2<0.001$		
FCM		<0.001 (0.001) $p=0.561$ $X^2=0.169$	
Calf T			0.008 (0.017) $p=0.512$ $X^2=0.215$

(c) Within-individual

	FAM	FCM	Calf T
FAM	**0.782** **(0.047)** **$p<0.001$** **$X^2=12.253$**	0.039 (0.040)	-0.003 (0.150)
FCM	0.030 (0.031) $p=0.203$ $X^2=0.809$	**0.762** **(0.043)** **$p<0.001$** **$X^2=11.543$**	-0.102 (0.142)
Calf T	-0.001 (0.060) $p=0.971$ $X^2=0.001$	-0.041 (0.057) $p=0.502$ $X^2=0.225$	**0.209** **(0.039)** **$p<0.001$** **$X^2=21.695$**

(2)LATE

(a) Among-individual

	FAM	FCM	Calf T
FAM	0.002 (0.019) $p=0.976$ $X^2<0.001$		
FCM		0.014 (0.019) $p=0.500$ $X^2=0.227$	
Calf T			

(b) Among-year

	FAM	FCM	Calf T
FAM	0.001 (0.002) $p=0.969$ $X^2=0.001$		
FCM		<0.001 (0.001) $p=0.563$ $X^2=0.167$	
Calf T			0.015 (0.026) $p=0.230$ $X^2=0.721$

(c) Within-individual

	FAM	FCM	Calf T
FAM	**0.783** **(0.047)** **$p<0.001$** **$X^2=12.994$**	0.038 (0.039)	-0.166 (0.142)
FCM	0.029 (0.031) $p=0.351$ $X^2=0.436$	**0.762** **(0.043)** **$p<0.001$** **$X^2=11.558$**	-0.118 (0.129)
Calf T	-0.068 (0.059) $p=0.292$ $X^2=0.556$	-0.047 (0.042) $p=0.361$ $X^2=0.418$	**0.214** **(0.038)** **$p<0.001$** $X^2=23.792$

***Table F. 3**: Fixed effects from bivariate mixed model examining the main effects of season and state on individual variation in female (a) FAM and (b) FCM concentrations amongst samples with known collection and freezing times.*

FIXED EFFECTS	**(a) FAM (484)**			**(b) FCM (576)**		
	Var. ± SE	*p*		Var. ± SE	*p*	
Age	-0.005 ± 0.038	0.636		0.033 ± 0.031	0.012	*
Age of final sample	0.008 ± 0.039	0.956		-0.022 ± 0.031	0.516	
February [a]	-0.225 ± 0.244			-0.025 ± 0.245		
March [a]	-0.096 ± 0.254			0.286 ± 0.258		
April [a]	-0.309 ± 0.267			0.581 ± 0.269		
May [a]	0.068 ± 0.348			0.936 ± 0.367		
June [a]	-0.091 ± 0.318			0.804 ± 0.323		
July [a]	-	0.001	**	0.776 ± 0.851	<0.001	***
August [a]	-0.230 ± 0.239			0.170 ± 0.232		
September [a]	-0.177 ± 0.226			0.418 ± 0.227		
October [a]	-0.138 ± 0.259			0.335 ± 0.259		
November [a]	-0.105 ± 0.300			0.141 ± 0.288		
December [a]	-			-		
Pregnant (late) [b]	0.259 ± 0.123	0.060	.	0.008 ± 0.128	0.927	
Lactating (♀ calf) [c]	-0.102 ± 0.098	0.568		0.029 ± 0.088	0.039	*
Lactating (♂ calf) [c]	0.037 ± 0.100			0.243 ± 0.092		
Assay date	*8 factor levels*	<0.001	***	*8 factor levels*	<0.001	***
Time to freezing	0.001 ± <0.001	0.014	*	<0.001 ± <0.001	0.526	
Age:Pregnant (late) [b]	-	-		0.049 ± 0.025	0.047	*

[a] monthly variance estimates are relative to January estimates

[b] pregnancy status relative to non- & early- pregnant females

[c] lactation status relative to non-lactating females

Table F. 4: *Fixed effects from linear mixed models examining the main effects of season and state on inter-individual variation in female (a) FAM and (b) FCM concentrations after correcting for temporal autocorrelation.*

FIXED EFFECTS	(a) FAM (611)			(b) FCM (735)		
	Est. ± SE	*p*		Est. ± SE	*p*	
Age	-0.012 ± 0.017	0.491		0.059 ± 0.015	<0.001	***
Age of final sample	0.013 ± 0.016	0.419		-0.046 ± 0.015	0.002	**
February [a]	-0.086 ± 0.215			0.063 ± 0.200		
March [a]	0.030 ± 0.225			0.241 ± 0.210		
April [a]	-0.106 ± 0.238			0.484 ± 0.223		
May [a]	0.385 ± 0.300			0.780 ± 0.299		
June [a]	0.176 ± 0.273			0.864 ± 0.267		
July [a]	0.148 ± 0.371	<0.001	***	0.758 ± 0.338	<0.001	***
August [a]	0.080 ± 0.206			0.196 ± 0.186		
September [a]	-0.030 ± 0.196			0.422 ± 0.180		
October [a]	-0.003 ± 0.228			0.250 ± 0.213		
November [a]	0.002 ± 0.253			0.133 ± 0.235		
December [a]	-0.194 ± 0.851			1.102 ± 0.833		
Pregnant (late) [b]	0.270 ± 0.119	0.024	*	0.004 ± 0.117	0.971	
Lactating (♀ calf) [c]	-0.105 ± 0.092	0.253		-0.001 ± 0.080	0.988	*
Lactating (♂ calf) [c]	-0.058 ± 0.093	0.531		0.234 ± 0.084	0.006	
Assay date	*8 factor levels*	<0.001	***	*8 factor levels*	<0.001	***
Time to freezing	<0.001 ± <0.001	0.005	**	<0.001 ± <0.001	0.329	
Age:Pregnant (late) [b]	-	-		0.057 ± 0.023	0.012	**

[a] monthly variance estimates are relative to January estimates

[b] pregnancy status relative to non- & early- pregnant females

[c] lactation status relative to non-lactating females

Table F. 5*: Temporal autocorrelation structure and p-values from nested ANOVA tests comparing the models presented in Table F. 4 with and without the autocorrelation structure.*

	Sample size	No of groups (ID)	Phi	Phi CI	*p*
FAM	611	187	0.174	0-1	0.965
FCM	735	189	0.204	0.007-0.898	0.555

Table F. 6: *Fixed effects for neonatal testosterone from two different multivariate mixed models. Neonatal testosterone concentrations were only included in this analysis if they were collected from calves born to mothers with faecal samples collected whilst pregnant with that calf. Where females were sampled more than once during pregnancy with the same calf, the last sample collected during (a) early and (b) late pregnancy (≤/> 118 days into gestation) was associated with calf testosterone levels in the within-individual covariance/correlation analyses. See Table 6.1 for fixed effects for FAM and FCM which do not differ between the two models. Full analysis of the complete neonatal testosterone dataset can be seen in chapters 3 & 4.*

FIXED EFFECTS	(a) Early (73)			(b) Late (76)		
	Var. ± SE	*p*		Var. ± SE	*p*	
After 24 hours	-0.040 ± 0.122	0.245		0.033 ± 0.031	0.279	
Calf age (hours) < 24 hours	-0.008 ± 0.002	<0.001	***	-0.008 ± 0.002	<0.001	***
Calf age (hours) >24 hours	-0.003 ± 0.001			-0.003 ± 0.001		
Sex (male)	0.194 ± 0.117	0.701		0.157 ± 0.115	0.624	
Collection time	0.018 ± 0.020	0.365		0.030 ± 0.019	0.086	.
Assay date	*6 factor levels*	<0.001	***	*6 factor levels*	<0.001	***

Table F. 7: *Bivariate mixed model estimating (a) among-individual and (b) within-individual variance components between female (n=768) and male (n=178) faecal cortisol metabolite concentrations (after being standardised for sex-specific variance). SE estimates are in parentheses. This model was significantly better than a model in which among-individual variances were constrained to be the same in both sexes ($\chi^2_{(1)}$=3.583; p=0.007).*

(a) Among-individual

	F	M
F	0.005 (0.021)	
M		0.210 (0.103)

(b) Within-individual

	F	M
F	0.995 (0.051)	
M		0.792 (0.103)