INVESTIGATING POPULATION PERFORMANCE AND FACTORS THAT INFLUENCE REPRODUCTIVE SUCCESS IN THE EASTERN BLACK RHINOCEROS (*DICEROS BICORNIS MICHAELI*)

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by

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ABSTRACT

Investigating population performance and factors that influence reproductive success in the eastern black rhinoceros (*Diceros bicornis michaeli*).

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With fewer than 5000 black rhinoceros remaining, *ex situ* populations play a vital role in the conservation of this species. To reinforce *in-situ* conservation efforts, captive populations must be self-sustaining, both demographically and genetically, to maximise future viability. The aim of this thesis was to determine the sustainability of the European captive population of eastern black rhinoceros, and investigate factors that may influence population performance in this species.

Population viability analysis of demographic data from *in situ* managed black rhinoceros populations were used to establish the variability in population growth rates across reserves. Secondly, these data were used to calculate fecundity, mortality and population structure performance indicators, as a reference for how this species can perform under natural conditions. The same analyses were then applied to the European *ex situ* population, to determine whether the population is demographically and genetically self-sustaining, and identify areas for potential improvement. The European captive population of eastern black rhinoceros, although currently self-sustaining, is performing sub-optimally both with respect to their *in situ* counterparts, and to a proposed target of 5% growth per annum. Population performance is primarily limited by sub-optimal reproduction, both in terms of individuals producing fewer calves per annum, and due to a high degree of reproductive skew across the population, leading to a large proportion of individuals failing to produce offspring.

A multi-institutional study was conducted on 90% of the European population, to investigate intrinsic differences in faecal reproductive hormone metabolites between breeding and non-breeding individuals. In females, irregular oestrous cyclicity was observed, with longer than average cycles observed more frequently in females that had never bred, and periods of acyclicity more common in females that had not bred for at least seven years. Non-proven females also had higher body condition scores, and were less likely to exhibit regular signs of oestrus. In males, non-breeding males had reduced faecal testosterone compared to males that had previously sired offspring. Extrinsic factors were also investigated, to determine whether differences in reproductive success could be attributed to aspects of the social or physical environment. However, no consistent relationships were observed between breeding and non-breeding males or females. Furthermore, adrenal activity and testosterone concentration were not correlated with environmental factors, indicating that extrinsic factors alone may not explain differences in reproductive success. However, within females, differences in hormone concentration were associated with irregular oestrous cyclicity. Long cycles were associated with increased glucocorticoid metabolite concentration, and oestradiol metabolites were lower during periods of acyclicity. The duration of the preceding luteal phase varied between cycle types, indicating that the occurrence of regular and irregular oestrous cyclicity may be influenced by hormone exposure during the preceding oestrous cycle.

In summary, a number of intrinsic differences in reproductive hormones in both males and females have been identified, which may be related to differential reproductive success. A better understanding of the causes of these differences would be beneficial to maximise growth rates and overall population performance of this *ex situ* population.

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List of Abbreviations

ABP	Androgen-binding protein
ABTS	2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)
ACTH	Adrenocorticotrophic hormone
AfRSG	African Rhino Specialist Group
AVP	Arginine vasopressin
BCS	Body condition scoring
BIAZA	British and Irish Association of Zoos and Aquariums
BSA	Bovine serum albumen
BSR	Birth sex ratio
CRH	Corticotropin releasing hormone
CV	Coefficient of variation
E_2	Oestradiol
EAZA	European Association of Zoos and Aquaria
EEP	European Endangered Species Breeding Program
EIA	Enzyme immunoassay
fE_2	Faecal oestradiol metabolite
fGCM	Faecal glucocorticoid metabolite
fPG	Faecal progesterone metabolite
FSH	Follicle stimulating hormone
fTt	Faecal testosterone metabolite
GC	Glucocorticoids
GD	Genetic diversity
GLMM	Generalised linear mixed model
GnRH	Gonadotropin-releasing hormone
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IBI	Inter-birth interval
ID	Identity
IGF-1	Insulin-like growth factor
IgG	Immunoglobulin G
IQR	Interquartile range
ISWE	International Society of Wildlife Endocrinology
IUCN	International Union for Conservation of Nature
KIFARU©	Kenya Black Rhino Information Management System
KWS	Kenya Wildlife Service
LH	Luteinising hormone
MeCN	Acetonitrile
MeOH	Methanol
NSB	Non-specific binding
PG	Progesterone
PMP	Population Management Plan
PVA	Population viability analysis
RCVS	Royal College of Veterinary Surgeons

RIA	Radio immunoassays
RMG	Rhino management group
s.e.m	Standard error of the mean
SADC	Southern African Development Community
SB	Studbook
SD	Standard deviation
SE	Standard error
SPARKS	Single Population Animal Record Keeping System
SSP	Species Survival Plan
Tt	Testosterone
UV	Ultra violet
VSS	Variance stabilised sensitivity analysis
WAZA	World Association of Zoos and Aquariums

CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

Captive breeding programs are an important component of *ex situ* conservation for a wide variety of species. However, in order for these populations to successfully fulfil their role, it is important that they are self-sustaining and managed cooperatively and effectively, to maximise their potential. Furthermore, animal managers have a duty of care to provide optimal conditions for welfare. There is increasing pressure on population managers both *in situ* and *ex situ* to maintain healthy and viable populations, and a number of tools are available to guide decision making for such managed populations. In particular, population viability analysis can be used to predict the likely future status of a population based on demographic parameters such as mortality and fecundity, and allow identification of areas of sub-optimal performance that may require targeted management. However, in order to provide the conditions required for optimal growth of a population, a better understanding of the factors that may influence population performance is often required. In this thesis, this approach will be utilised to investigate population performance and factors that influence reproductive success in the eastern black rhinoceros (*Diceros bicornis michaeli*). Although this thesis will focus upon the black rhinoceros, the methodologies involved could be applied to a variety of taxa, to investigate similar issues regarding population viability, factors that may influence population performance and specifically reproduction and differential reproductive success.

1.1. *Ex situ* conservation

1.1.1. The importance of species conservation

Although extinction is a naturally occurring process, as evidenced by the fossil record, the current rate is elevated due to anthropogenic influences such as destruction of habitats for farming or human habitation, over-exploitation of natural resources, introduction of alien species, pollution and climate change, and harvesting of animal and plant species at unsustainable levels either for food, the pet trade or for traditional medicines. According to the International Union for Conservation of Nature (IUCN), the current species extinction rate is estimated between 1,000 and 10,000 times higher than it would naturally be (IUCN 2012). Of the 25,780 species categorised on the IUCN red list in 2010, one-fifth of these were threatened with extinction (Hoffmann et al. 2010), a figure that continues to increase, with on average 52 species of mammals, birds and amphibians moving one category closer to extinction each year. However, without the work of conservation organisations around the world, extinctions would be at least one fifth higher than current figures. However, many of the species at risk today have become conservation dependent, such as 84% of those listed under the US endangered species act (Scott et al. 2010), and with an ever-increasing list, there is a need for evidence based conservation (Sutherland et al. 2004), to ensure that resources can be best applied.

Although conservation may be most effective within a species' natural habitat (Redford et al. 2012), with the added benefit that conservation work focused upon so-called 'umbrella species' can actually be beneficial in preserving ecosystems, this approach is not always feasible. For example, if the threat of disease remains, as is the case of the amphibian fungal disease *Chytridiomycosis* (Zippel et al. 2011), it may not be safe to conserve species *in situ* while the source of population declines still exists. Therefore *ex situ* conservation can be used in conjunction, to provide a safe and secure back up population, whilst attempting to tackle the cause of species declines. Although there has been some debate over the use of captive breeding programmes (Bowkett 2009), the consensus seems to be that the best approach to global conservation is through a combination of *in situ* and *ex situ* techniques (Balmford et al. 2011; Conde et al. 2011a, b; Pritchard et al. 2012). Indeed, a cooperative *in situ-ex situ* metapopulation, with the potential for future movement between the two if required, may indeed be the best approach to endangered species management (Lacy 2013).

1.1.2. Captive breeding programs

Ex situ populations can contribute to global conservation strategies in a number of ways, both for the species involved, and for wider conservation initiatives (Baker 2007; IUCN 2002; WAZA 2005). Through the establishment of captive populations, away from the threats experienced *in situ*, a demographic and genetic reservoir can be established. If a founder population is established that is a good representation of their wild counterparts, with careful management, a high level of genetic diversity can be maintained away from the potential dangers experienced in the wild. Particularly for species that have undergone habitat loss and fragmentation, or where only small isolated populations remain, genetic diversity can be maximised through breeding management, and may allow a higher retention than leaving animals *in situ* where limited mixing may be feasible. Additionally, for species that have been pushed to the brink of extinction, removing vulnerable individuals to a safe captive environment, where for example, disease risk can be minimised, can provide a buffer against further decline while population breeding and recovery programs can be initiated.

Furthermore, in situations where species have the potential for future reintroduction and suitable habitat remains, captive breeding can provide a means whereby proliferation can occur *ex situ.* Successful captive breeding programs have been used to re-establish populations of a number of species including the black footed ferret (*Mustela nigripes;* (Jachowski and Lockhart 2009; Vargas et al. 1998)), golden lion tamarin (*Leontopithecus rosalia;*(Kierulff et al. 2012)), California condor (*Gymnogyps californianus;* (Walters et al. 2010)), Przewalski's horse (*Equus ferus przewalskii;* (Ryder 1993)), and red wolf (*Canis rufus;* (Phillips et al. 2003)).

Whilst species are maintained in captivity, they can also provide other opportunities, including a chance to conduct research in a controlled environment that can then be applied to conservation *in situ*. Areas of research such as the effect of inbreeding (Ralls et al. 1988), can not only be beneficial for *ex situ* conservation, but can also be applied *in situ* (Redford et al. 2012; Seddon et al. 2007) to improve management of populations. Similarly, controlled conditions in a captive environment enable us to learn about species biology, that would be difficult to achieve under natural conditions (Watts et al. 2006). Research can also be beneficial when little is known about a species' requirements, so that the correct conditions can be provided *ex situ*, but also to guide conservation priorities *in situ*, for example to determine social relationships or dietary requirements, which can have an impact on required reserve size. In other

cases, where disease outbreaks may be to blame for species decline, it may be necessary to research the potential causes and develop treatment before any reintroduction programme could be put into place.

Flagship species in zoos and aquaria also act as important ambassadors to educate, and increase both public awareness of conservation issues, as well as acting as catalysts for local involvement (Baker 2007). Zoological institutions have a tremendous potential to educate the public about conservation issues (Moss and Esson 2013), both in the form of educational programmes, and by demonstrating the fantastic biodiversity that exists in real and tangible terms, giving people more of a connection to the issues we face. Zoological institutions do not only contribute to raising awareness of global conservation issues, but also raise financial support that can be used to support *in situ* conservation (Gusset and Dick 2011), indeed many zoos have direct links to *in situ* initiatives to which both expertise and financial support are provided.

Ex situ populations have an important role to play in global conservation strategies, which with the current rate of extinction, may become even more important in the future, which makes it vital that a science-based approach is taken towards conservation, to ensure the best use of finite resources.

1.1.3. Population management

However, to fulfil these goals, captive populations must be self-sustaining, often without supplementation from the wild (Lees and Wilcken 2009, 2011), and populations must be managed scientifically, and cooperatively, to ensure their long-term viability (Foose 1980; Foose and Wiese 2006; Leus et al. 2011b). To support conservation efforts, coordinated captive breeding programs including the European Endangered Species Breeding Program (EEP) in Europe and the Species Survival Plan (SSP) and Population Management Plan (PMP) in America have been established for a wide variety of species (Baker 2007). These coordinated programs mean that although individuals may be physically separated at multiple institutions, they can be managed as a single population, thereby increasing the potential sustainability of captive breeding programs. One particular concern of cooperative breeding programs is to maintain healthy, demographically sustainable populations with sufficient genetic diversity for future viability (Lacy 2013).

Captive breeding programs are often limited both in terms of the number of founders, and total population size (Lacy 2013). However, to act as a viable reserve for endangered species, it is important that *ex situ* populations are genetically sustainable, to retain the natural characteristics representative of their *in situ* counterparts (McPhee and Carlstead 2010) and sufficient genetic diversity for individuals to survive and reproduce, while the population maintains the potential to adapt to future changes in the environment, without becoming adapted to captivity (Frankham 2008; Williams and Hoffman 2009). A population is generally considered to be sustainable in the long-term, according to certain criteria: reproduction should at least equal mortality; populations should be demographically stable, with 95-99% probability of population survival over a given time period; and genetic diversity should be maintained above 90% for 100 years (Amin et al. 2006; Foose et al. 1995; WAZA 2005).

However, more recently, it has been suggested that many cooperative breeding programs are failing to reach these targets (Conway 2011; Leus et al. 2011a; Long et al. 2011), and these initial criteria may not be sufficiently strict to preserve the viability of *ex situ* populations in the longer term (Lacy 2013). An initial founder population of 20 individuals can be sufficient to achieve the specified 90% genetic diversity for 100 years (Lacy 1989; Soule et al. 1986). However, this calculation was based on effective population size, and in reality, founder contribution is often uneven, meaning that a minimum of 30-50 founders is often required to achieve an effective population of 20, and retain the necessary level of diversity (Lees and Wilcken 2009; Leus et al. 2011a). An important aspect of captive management is therefore to minimise reproductive skew, ensuring that all founders are well represented within the population (Ballou et al. 2010), to slow the rate of genetic change. For some species, it may also be necessary to manage *ex situ* populations globally, and even exchange individuals with *in situ* populations, taking a metapopulation approach (Conway 1995; Lacy 2013; Stanley-Price and Fa 2007) in order to achieve these goals.

To maximise the conservation potential of *ex situ* populations, it is vital to understand the factors that may influence current and future viability. Population viability analysis (PVA) is a useful management tool in conservation biology, which uses quantitative methods to predict the likely future status of a population, and can be applied to both *in situ* (Carrete et al. 2009; Daleszczyk and Bunevich 2009) and *ex situ* populations (Faust et al. 2006; Faust et al. 2003) to assess population performance, and investigate effective management strategies. PVA can be used to estimate the likely future growth

rate of a population (Wittmer et al. 2010), the risk of extinction over a given time period (Lee et al. 2011), or the time required to reach a target population size (Earnhardt et al. 2001). PVA can also guide management decisions, by quantifying the relative contribution of particular groups of individuals to overall population growth (Dunham et al. 2008; Fernandez-Olalla et al. 2012), enabling targeted management and making PVA an integral part of species management (Boyce 1992). For example if such analyses indicate that reduced fecundity is limiting population growth, it is then important to understand the reproductive physiology of the species in question, to guide breeding management.

1.2. Endocrine control of reproduction

In order to maximise population performance, an understanding of how intrinsic and extrinsic factors that influence reproduction may be required, so that optimal conditions can be provided. However, before species differences in reproductive function or sub-optimal performance can be investigated, an understanding of the endocrine control of reproduction is first required. The endocrine system is one of the main control systems within the body, which acts through the production of hormones. Hormones are chemical substances produced by specialised cells, acting as messengers to communicate with target cells throughout the body. In both males and females, reproduction is controlled by series of hormones from the hypothalamic-pituitary-gonadal (HPG) axis, including the hypothalamus and higher brain centres, the pituitary gland and either the testes or ovaries in males and females respectively.

1.2.1. Female mammals: the oestrous cycle

Once a female reaches puberty, she enters into a period of cyclicity that may last her entire reproductive life. This period of cyclicity, termed the oestrous cycle, varies in length depending on the species, ranging from as short as 4-6 days in the house mouse (*Mus musculus*) (Parkening et al. 1982) to 14-16 weeks in the elephant (*Elephas maximus, Loxodonta africana*) (Hodges 1998), and is defined as the time from one period of receptivity, known as oestrus, to the next. Oestrus is often the only period of the cycle when the female is receptive to the male, and when ovulation occurs. This cyclicity is controlled by a series of hormones from the hypothalamic-pituitary-ovarian

axis (Figure 1.1) (Johnson and Everitt 2000). The primary hypothalamic hormone of the reproductive system is gonadotropin release hormone (GnRH), which is pulsatile, and stimulates the pituitary gland to release the gonadotropins follicle stimulating hormone (FSH) and luteinising hormone (LH). FSH controls the development of the gametes, whereas LH controls ovulation and post-ovulatory changes in the female.

FSH allows the development of a cohort of oocytes, which split from the germinal epithelium and migrate in to the body of the ovary. They become surrounded by theca cells, which when bound by LH, produce oestrogens. Prolactin is released from the pituitary gland in response to the elevation in oestrogens, and FSH converts oestrogens into oestradiol. Prolactin has a twofold influence on follicular development, both increasing LH production, and the number of LH receptors present on the follicles. Recruitment of a new cohort of follicles occurs when FSH levels are high. Follicular development occurs continuously throughout the oestrous cycle, but the fate of the follicles depends on the timing of LH production.

The release of LH from the pituitary is pulsatile, and the frequency of these pulses is the critical factor as to whether a wave of follicles will progress or undergo atresia. A high LH pulse frequency will result in the production of androgens, to be converted to oestradiol, a low frequency will not result in androgen production, and the oocytes will degenerate. If the follicles remain, a dominant follicle will emerge, as FSH levels decrease. The dominant follicle becomes less reliant on FSH, due to growth factors such as insulin-like growth factor IGF-1, and continues to mature while the other follicles undergo atresia. The oestradiol produced initially has a negative feedback effect on GnRH, allowing the build-up of LH in the pituitary gland. Oestradiol then switches to having a positive feedback, resulting in the LH surge. The follicle moves to the surface of the ovary, and the LH surge caused by prolactin and oestradiol then results in an influx of sodium, and consequently the inflow of water through osmosis. This causes an increase in pressure, resulting in ovulation, as the oocyte bursts from the follicle.

Once the follicle has ruptured, local haemorrhage forms a structure known as the corpus haemorrhagicum. Luteinisation of the theca and granulosa cells then results in the formation of the corpus luteum on the ovary at the site of ovulation. The LH peak also stimulates the corpus luteum to convert oestradiol to progesterone and begin producing progesterone. Progesterone has an inhibitory effect on GnRH, suppressing any further release of gonadotropins. If fertilisation has occurred, the corpus luteum may remain for the length of pregnancy, producing progesterone along with the

placenta, and preventing the resumption of cyclicity. The corpus luteum also produces oxytocin, which if no conception has occurred, stimulates the production of prostaglandin F2α from the uterus, which inhibits progesterone and allows the resumption of cyclicity. Prostaglandin F2α also stimulates further oxytocin, and a positive cascade results in yet more prostaglandin F2α to reduce progesterone levels rapidly. As progesterone levels drop markedly, the restraint of GnRH is released, and LH/FSH production resumes. A new wave of follicles now emerges, and the cycle begins again.

All the hormones produced by the pituitary gland (LH, FSH, prolactin and oxytocin), in combination with the hormones from the gonads (oestrogens, progesterone, inhibin, oxytocin and relaxin), work in a positive and negative feedback loop to the hypothalamus, pituitary gland, and the higher brain centres, to regulate production of hormones, and ultimately control reproductive function. The oestrous cycle depends on the correct pattern of hormone secretion, at the required time and the necessary concentrations. Disruption at any point of the cycle can result in a failure to ovulate. In non-seasonal breeders, cyclicity should only be suspended by pregnancy and lactation. However, the reproductive system is susceptible to disruption, and is one of the first systems to shut down under periods of stress or imbalance of homeostasis (Schatten and Constantinescu 2007).

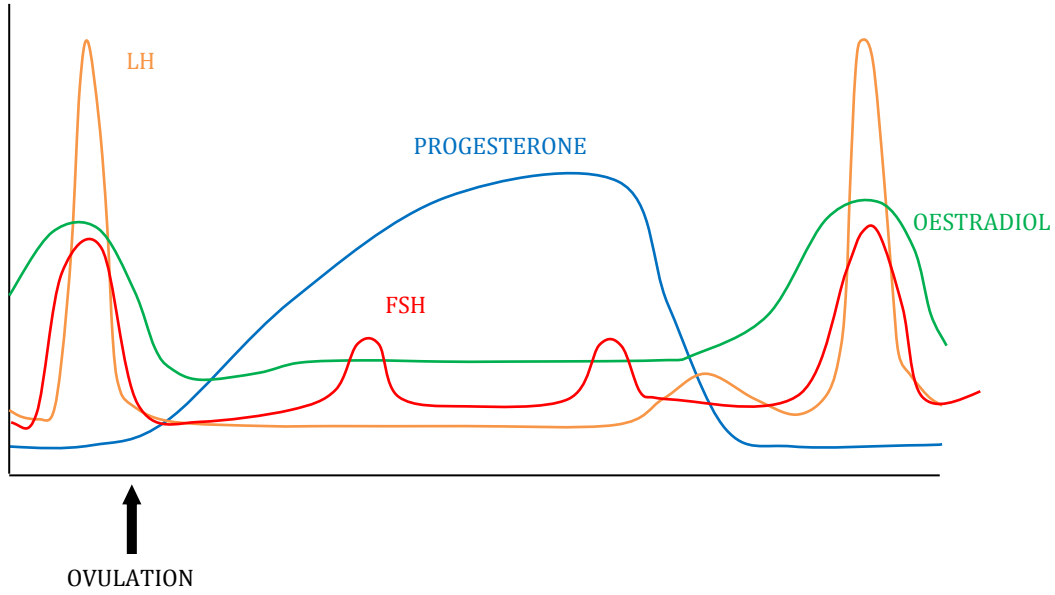


Figure 1.1: Schematic diagram illustrating the relative secretion of pituitary gonadotropins LH and FSH and ovarian steroid hormones oestradiol and progesterone during the oestrous cycle, relative to ovulation.

1.2.2. Endocrine control of oestrus

Concurrent with the final growth of the dominant follicle, an increase in oestradiol production also prepares the body for oestrus. This increase in oestradiol concentration, in the relative absence of progesterone, acts on the hypothalamus to induce oestrus behaviours (Allrich 1994). The particular behaviours involved in proceptivity (proactive behaviour to increase the chances of mating) and receptivity (responsiveness to those proceptive behaviours from a potential mate) are species specific, as is the duration of expression. However the hormones involved in inducing sexual behaviour appear to be relatively consistent across species.

In ovariectomised cows (Asdell et al. 1945; Carrick and Shelton 1969; Katz et al. 1980), sheep (Fabre-Nys and Martin 1991a; Fabre-Nys et al. 1993), horses (Asa et al. 1984), and pigs (Ford 1985), oestrus has been induced using oestradiol. However, the effect of oestradiol in inducing oestrus seems to be an 'all or nothing' scenario, where behaviours are not expressed until a certain threshold has been reached (Allrich 1994). Progesterone is inhibitory to the expression of oestrus behaviour (Asa et al. 1984;

Davidge et al. 1987; Fabre-Nys and Martin 1991b; Ford 1985), and concentrations must be low during proestrus, or oestrus will be suppressed regardless of oestradiol concentration (Vailes et al. 1992). However, prior exposure to progesterone may facilitate the expression of oestrus (Asa et al. 1984; Fabre-Nys and Martin 1991b; Melampy et al. 1957), and lower prior progesterone exposure may be related to reduced intensity of oestrus (Walker et al. 2008).

1.2.3. Male mammals: spermatogenesis

As in the female system, the hypothalamus, pituitary gland and gonads play a key role in reproduction of male mammals (Johnson and Everitt 2000). The testis, made up of Leydig cells and Sertoli cells, also then influence the accessory glands, and negative feedback prevents overloading of the system. Hormone production is low prior to puberty, but thereafter spermatogenesis is often continuous.

Initiation of spermatogenesis is largely reliant on the concentration of the hormones testosterone and follicle stimulating hormone (FSH). Luteinising hormone (LH) is released in pulses from the pituitary gland, in response to gonadotropin release hormone (GnRH) pulses from the hypothalamus. In the male, LH stimulates the Leydig cells to produce testosterone, and controls development of secondary sexual characteristics. Testosterone then feeds back to the hypothalamus to prevent GnRH secretion, and further FSH and LH release from the pituitary. FSH stimulates conversion of testosterone to oestradiol, and promotes inhibin and androgen-binding protein (ABP) production. Oestradiol also has a negative feedback effect on GnRH from the hypothalamus. The primary spermatogonia divide to produce two daughter cells. One of these returns to the germinal epithelium to replace the primary spermatogonia, while the other divides further to produce sperm. After the final division, some residual cell mass remains, and is taken up by the Sertoli cells. This residual cell mass is high in cholesterol, and as this builds up, androgen-binding protein is inhibited, and inhibin is produced to prevent gonadotropin release. Spermatogenesis is reduced, and cholesterol levels decrease, resulting in an increase in ABP production, and resumption of the cycle.

As a male cannot predict when a female will come into oestrus, it is important for the male to be continuously fertile, or within the breeding season in seasonally breeding

species. Unless influenced by season, spermatogenesis is therefore continuous, despite the cyclicity of GnRH and gonadotropin release. Continuous sperm production is possible by an uneven distribution of Sertoli cells across the testis, meaning that only around a third of the cells are active at any one time.

1.3. Factors that can lead to disruption of reproduction

Reproduction may be disrupted under normal circumstances, for example in seasonal breeders, where reproduction only occurs during part of the year. Alternatively, females will cease their normal pattern of cyclicity during pregnancy and in many cases during lactation. However, there may be incidences where infertility is not a normal process, but can be brought about due to physiological disruption.

1.3.1. Adrenal activity

When an individual experiences a threat to its homeostasis, the response to that potential stressor is organised into three stages; recognition, biological defence, and consequences of the stress response (Moberg 2000). Recognition by the central nervous system is followed by a combination of autonomic nervous system, behavioural, immune and neuroendocrine responses, that will lead to altered biological function. If a behavioural response of avoidance is not feasible or sufficient, the autonomic nervous system may be activated, allowing a 'fight or flight' response (Cannon 1929), resulting in changes in heart rate, blood pressure, or gastrointestinal activity. The main neuroendocrine response to a potential stressor involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Matteri et al. 2000). In response to a stimulus, corticotropin releasing hormone (CRH) is released from the hypothalamus, stimulating the pituitary to produce adrenocorticotrophic hormone (ACTH) and finally glucocorticoids such as cortisol and corticosterone from the adrenal gland (Figure 1.2). This response is regulated through positive and negative feedback mechanisms, allowing the body to respond accordingly to the perceived stimulus and maintain homeostasis (Moberg and Mench 2000).

A wide range of both positive and negative stimuli can elicit a stress response (Buwalda et al. 2012), including social (Creel 2001), physical (Neumann et al. 1998)

and physiological stressors (Gasparotto et al. 2005). The body's response to a certain stressor can also depend on a number of factors, including the genetics or the age of the individual (Moberg 2000), the physiological state of the individual (Nisenbaum et al. 1991) and how the stressor is perceived. This perception of the potential stressor may depend on the predictability of the stressor, the prior experience and the personality of the individual (Sapolsky 1994). The stress response itself is primarily an adaptive response, facilitating the mobilisation of energy stores (Moberg 2000), allowing the body to respond to changes in the environment (McEwen and Wingfield 2003). However, if the body is not able to cope appropriately with the stimulus, negative consequences can result, leading to disruption of other processes including the immune response (Khansari et al. 1990) and reproduction (Dobson et al. 2003; Dobson and Smith 2000), as priority is given to coping with the stress and restoring homeostasis. Consequently, a state of stress is commonly defined as a disruption of homeostasis (Rivier and Rivest 1991) or failure of an individual to cope with its environment (Dobson and Smith 2000), potentially leading to the development of pathology.

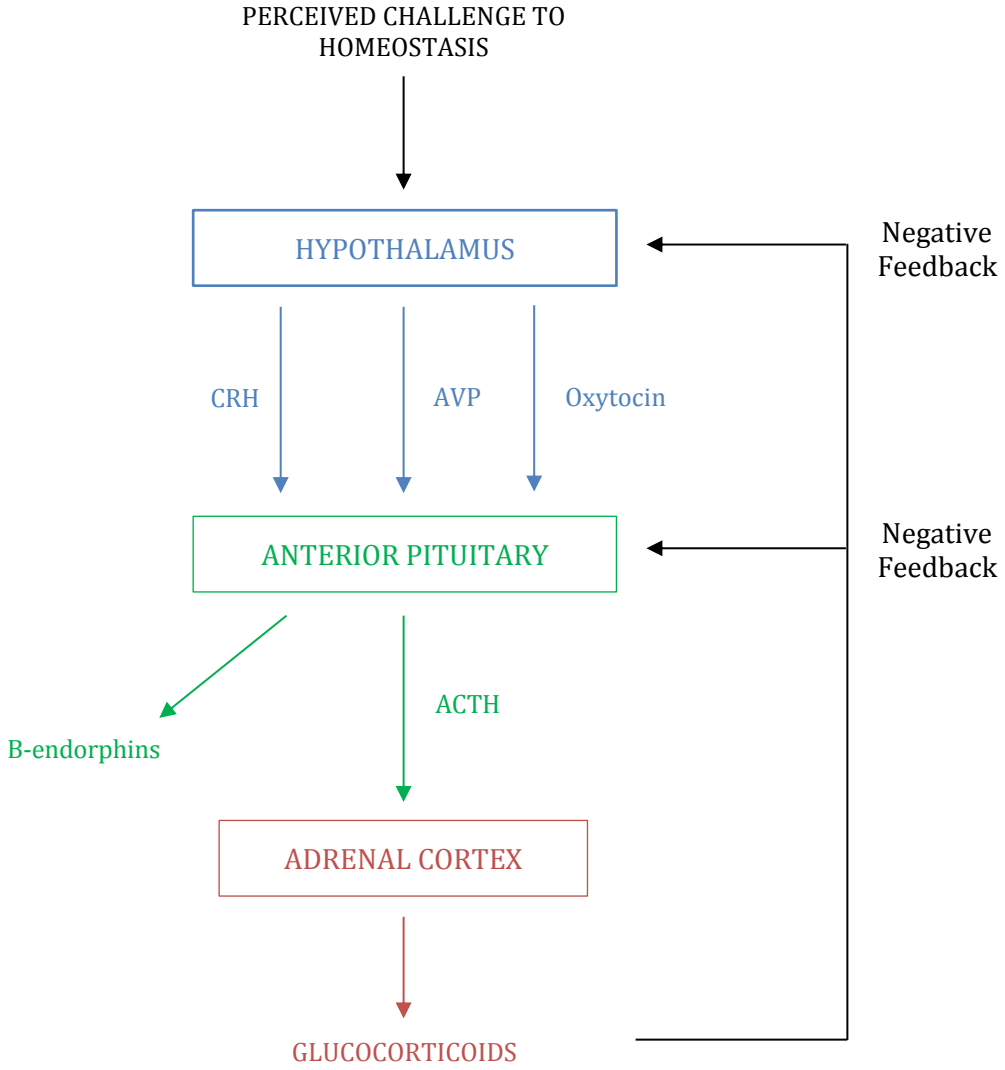


Figure 1.2: Schematic diagram of the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis in response to the perception of internal or external stimuli. Activation includes the production of corticotropin-releasing-hormone (CRH), arginine vasopressin (AVP) and oxytocin from the hypothalamus, adrenocorticotrophic hormone (ACTH) and β-endorphins from the anterior pituitary and glucocorticoids from the adrenal cortex. The system is regulated via negative feedback at the pituitary and hypothalamus. Figure adapted from Moberg and Mench (2000), and Wingfield and Sapolsky (2003).

1.3.2. Disruption of reproduction due to stress

Although a wide range of stressors have the potential to impact reproduction, all are alike in that disruption occurs through the interaction of the HPA and HPG axes (Uphouse 2011). Much of the research investigating the mechanisms by which disruption occurs has been conducted in rodents (Brann and Mahesh 1991; Rivier and Rivest 1991), domestic ungulates (Dobson et al. 2003; Dobson and Smith 2000; Tilbrook et al. 2002), primates (Olster and Ferin 1987; Tamashiro et al. 2005) and humans (Chrousos et al. 1998; Schenker et al. 1992), but differences in reproductive function associated with adrenal activity have also been observed in free-ranging populations, such as the effect of predation risk on reproduction in the 10-year cycles of snowshoe hare, *Lepus americanus* abundance (Boonstra et al. 1998), or sustained social stress on testicular function in olive baboons, *Papio anubis* (Sapolsky 1985). This process been also been addressed in a wide range of taxa including amphibians and reptiles (Carr 2011; Moore and Jessop 2003; Tokarz and Summers 2011), fish (Fuzzen et al. 2011; Schreck 2010) and birds (Breuner 2011). Furthermore, physical (Dobson and Smith 2000), behavioural (Moberg 1991), social (Sapolsky 1985), nutritional (Wade and Schneider 1992), and environmental (Boonstra et al. 1998) stressors have all been illustrated to disrupt reproduction through activation of the stress response.

Stress can disrupt reproductive function on all levels of the HPG axis (Dobson and Smith 2000; Rivier and Rivest 1991) (Figure 1.3), through the action of CRH and ACTH primarily on the hypothalamus and higher brain centres, while glucocorticoids may potentially act on every level of the HPG axis and associated reproductive structures (Wingfield and Sapolsky 2003). Females are particularly sensitive to disruption during the period prior to ovulation, as a delicate balance of hormonal events occurs, during which the precise timing is crucial (Dobson and Smith 2000). During this pre-ovulatory period, reproduction can be disrupted via inhibition of GnRH secretion from the hypothalamus, influencing both the frequency and amplitude of pulsatile secretion (Dobson et al. 2003). Inappropriate GnRH pulsatility then reduces LH release from the pituitary gland, perhaps further impacted by a direct inhibitory effect of glucocorticoids on the pituitary gland, making it less responsive to GnRH. Finally, glucocorticoids act on the ovaries, to decrease sensitivity to LH, perhaps through a reduction in receptor availability (Wingfield and Sapolsky 2003). In combination, this reduction of GnRH/LH pulsatility and decreased sensitivity can potentially have multiple effects. Firstly, insufficient GnRH/LH frequency may prevent follicular

development, leading to an extended follicular phase, and anoestrous (Dobson and Smith 2000). Another scenario may occur whereby pulse frequency is sufficient for normal follicular development, but not of sufficient strength to enable the final LH surge, and ultimately ovulation, to occur. This can result in a persistent follicle, known as cystic ovarian syndrome (Dobson and Smith 2000), or chronic anovulatory syndrome (Ferin 1999). Finally, ovulation may occur, but inappropriate priming of the oocyte could reduce viability, leading to a lower chance of successful establishment of pregnancy (Dobson and Smith 2000).

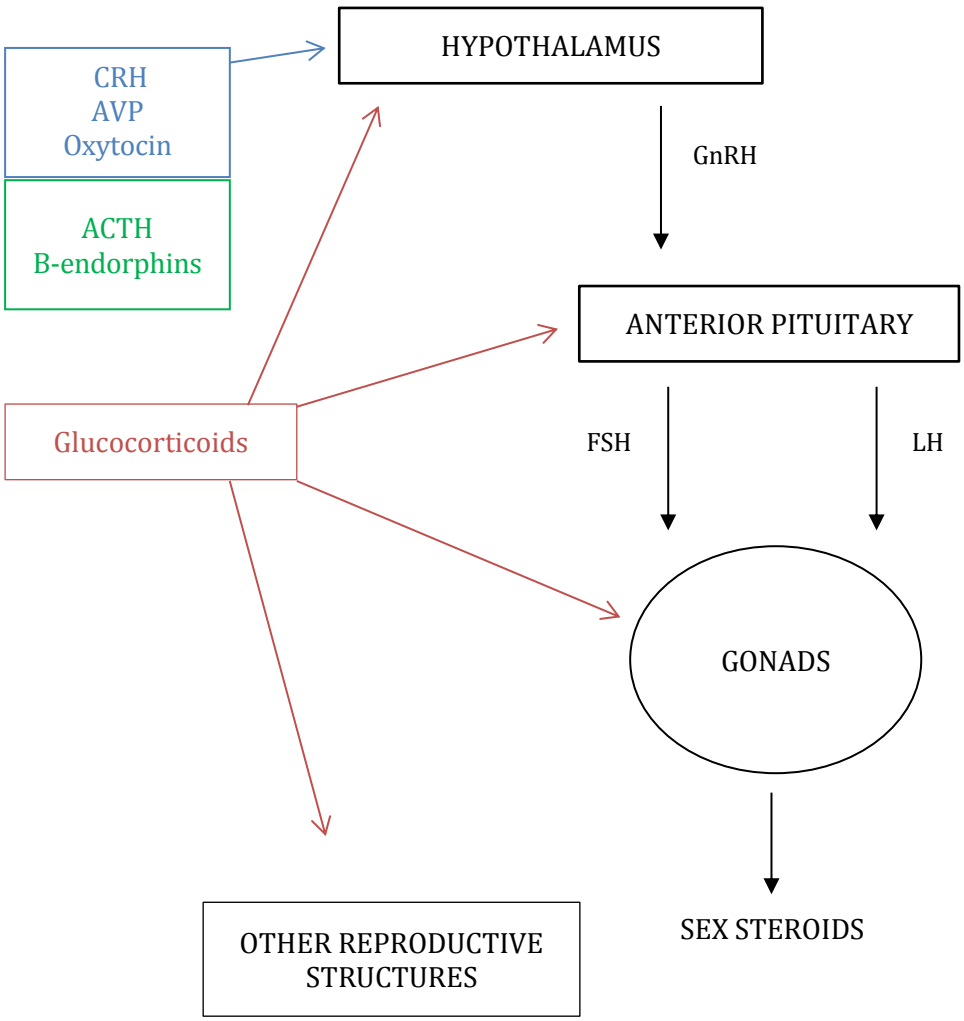


Figure 1.3: Schematic diagram of some of the ways in which stress can potentially disrupt the hypothalamic-pituitary-gonadal (HPG) axis. Hypothalamic and pituitary products of the HPA axis may primarily act upon the higher levels of the HPG axis, but glucocorticoids may act at all levels including other reproductive structures such as the uterus. Figure adapted from Wingfield and Sapolsky (2003).

In females, stress can also influence uterine growth and implantation success (Kalantaridou et al. 2004; Kalantaridou et al. 2010; Rabin et al. 1990), perhaps due to a decline in the progesterone required for uterine maturation, or via a stress-induced production of prolactin, which inhibits the actions of progesterone in the uterus (Wingfield and Sapolsky 2003). Even if conception does occur, stress can also play a role in early pregnancy failure (Einarsson et al. 1996; Parker and Douglas 2010). Furthermore, due to GnRH and gonadotropin insufficiency, biosynthesis of sex steroids can also be inhibited (Kalantaridou et al. 2010). As the expression of sexual behaviour is also controlled by the neuroendocrine system, disruption of sex steroids can impact the expression of proceptive (Carter 1992) and receptive behaviours in response to stress (Papargiris et al. 2011).

In males, HPA activity can similarly impact GnRH/LH pulsatility at the level of the hypothalamus and pituitary (Kalantaridou et al. 2010). Furthermore, increases in glucocorticoid concentration inhibit testosterone-biosynthetic enzyme activity (Orr et al. 1994), leading to a reduction in testosterone secretion (Hardy et al. 2005). There is also evidence that there may be a direct impact on fertility through reduction in the number, as well as the function of Leydig cells, perhaps through apoptosis, a form of controlled cell death (Hardy et al. 2005). Furthermore, severe psychosocial stress, in humans has been associated with reduced sperm count, perhaps related to the reduction in testosterone, but possibly also due to direct effects upon the seminiferous epithelium (Fenster et al. 1997). Cumulatively, these effects can lead to diminished libido and fertility (Phillips et al. 1989).

Although the interaction between the HPA and HPG axes are involved in disruption in both sexes, there may be differences in the mechanism by which stress interrupts normal function. One example was demonstrated in gonadectomised ewes and rams, subjected to isolation and restraint, and injected with GnRH (Tilbrook et al. 1999). The amplitude of LH pulsatility was reduced in rams, whereas frequency of pulses, but not amplitude was affected in ewes, indicating that there may have been a sex-difference in the mechanism and site of action by which stress was influencing reproduction (Tilbrook et al. 2000).

The effect of stress on reproduction can also be moderated by a number of factors, that may reduce the potential disruption on fertility (Wingfield and Sapolsky 2003). Differences in the relative cost of disruption in particular may influence whether an individual's reproduction is disrupted. For example, seasonal breeders which only have

a finite window of opportunity to breed may be more resistant to acute stressors (Boonstra et al. 2001). Similarly, in individuals that may have limited opportunity for breeding due to their age (Boivin et al. 2006) or their social status (Sapolsky 1985), if the costs of failed reproduction are higher than the potential costs of the stressor it may be beneficial to become resistant to stress. Additionally, there is some evidence to suggest that acute stressors may not always be inhibitory, but under certain circumstances could in fact stimulate reproductive function (Brann and Mahesh 1991; Rivier and Rivest 1991; Tilbrook et al. 2000).

1.3.3. Nutrition and body condition

Research investigating reproduction in both humans and domestic animals in particular, has indicated that either too low body condition or too high body condition can potentially inhibit reproduction. For example, in dairy cattle, too low body condition has been associated with ovulatory failure, whereas too high body condition may be related to impaired folliculogenesis, reduced oocyte quality and embryonic failure (Ferguson 2005). Similarly, in humans, under-nutrition can lead to delayed puberty and amenorrhea (Frisch and McArthur 1974), whereas obesity has been linked to a number of reproductive problems including oocyte development, ovulation, endometrial development, implantation, embryo development, and pregnancy loss (Brewer and Balen 2010; Norman 2010). Obesity has also been linked to fertility problems in males, as excess adipose tissue increases the conversion of testosterone to oestradiol, resulting in reproductive axis suppression and reduced testosterone concentration (Michalakis et al. 2013). Furthermore oxidative stress resulting from fat accumulation has also been linked to decreased spermatogenesis (Michalakis et al. 2013).

1.3.4. Health

Another factor that has been associated with reduced reproductive function, particularly in long-lived, slow breeding species such as the elephant and rhinoceros in captivity is that of asymmetric reproductive aging (Hermes et al. 2004). Long periods without reproduction can have detrimental effects on their reproductive system, leading to the development of pathologies, reduced fertility, and irreversible acyclicity

leading to premature senescence. It is thought that prolonged exposure to endogenous sex steroids during continuous cyclicity without conception play a role in this decline, as females that fail to reproduce early in their reproductive life may have already exhibited as many oestrus cycles by middle-age as a regularly breeding female would in her entire lifetime. In captive elephants, a non-breeding female's active reproductive lifespan may be shortened by as much as 15 years compared to that of a breeding female (Hildebrandt et al. 2000). The constant exposure to ovarian sex steroids have been linked to the development of reproductive pathologies, which together with the exhaustion of finite numbers of follicles, reduced chances of conception due to oocyte viability and uterine function, and reduced capability of corpora lutea to support early pregnancy, the chances of reproduction also decline with age (Hermes et al. 2004).

1.4. The black rhinoceros

1.4.1. Species information and distribution

The family *Rhinocerotidae* are odd-toed ungulates, which along with *Tapiridae* and *Equidae*, make up the order *Perissodactyla*. The ancestors of this family have been on Earth for approximately 60 million years (Bradley Martin and Bradley Martin 1982; Toon and Toon 2002), and were a diverse and wide ranging group. Throughout history there were numerous species of rhinoceros, with at least 30 genera, but the majority are now extinct; today only four genera and five species of rhinoceros remain. All of these remaining rhinoceros species are threatened with extinction; the most endangered being the Javan rhinoceros (*Rhinoceros sondaicus*; critically endangered) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*; critically endangered), followed by the black rhinoceros (*Diceros bicornis*; critically endangered), greater one-horned rhinoceros (*Rhinoceros unicornis*; vulnerable) and white rhinoceros (Southern *Ceratotherium simum simum*; near threatened and Northern *Ceratotherium simum cottoni;* critically endangered).

There are three remaining subspecies of black rhinoceros, ranging across central and southern Africa. The eastern black rhino (*D. b. michaeli*) used to range across eastern Africa, from southern Sudan and Ethiopia, through Somalia, Kenya, and Rwanda as far as northern Tanzania. The current stronghold of this species is Kenya, with a smaller population in Tanzania, and an out-of-range population in South Africa. The south-western black rhino (*D. b. bicornis*) is the largest subspecies, and lives in more arid

regions. The current stronghold of this subspecies is the desert and arid savannah regions of Namibia, with smaller populations also in South-western South Africa, and possibly a small number of animals in Angola. The most numerous of the remaining subspecies, the south-central black rhino (*D. b. minor*) historically occupied a large range from western and southern Tanzania in the north, down to eastern South Africa. This subspecies may also have been present as far west as northern Angola. The current stronghold of this species is South Africa, with individuals also remaining in Zimbabwe, and southern Tanzania. This subspecies has also been reintroduced to areas within its original range, including Botswana, Malawi, Zambia and Swaziland (Emslie 2012b; Emslie and Brooks 1999). The fourth subspecies, the western black rhino (*D. b. longipes*) native to the savannah zones of central-west Africa was declared extinct in 2011 (Emslie 2011).

1.4.2. Current conservation status

The black rhinoceros (*Diceros bicornis*) has been listed as critically endangered on the IUCN Red List of Endangered Species since 1996, following an estimated 97.6% decline in the wild population since 1960 (Emslie 2012b). The black rhinoceros was once numerous across central and southern Africa, with perhaps several hundred thousand individuals in the early nineteenth century (Emslie and Brooks 1999). However, unsustainable hunting and land clearance meant numbers were reduced to approximately 100,000 in 1960. By 1970 this had been further reduced to 65,000, and by 1995 the black rhino had been pushed to the brink of extinction, with only 2,412 individuals remaining (Emslie and Brooks 1999) (Figure 1.4). Illegal poaching for rhino horn was the main cause of this dramatic decline, and remains the greatest threat to rhinoceros populations.

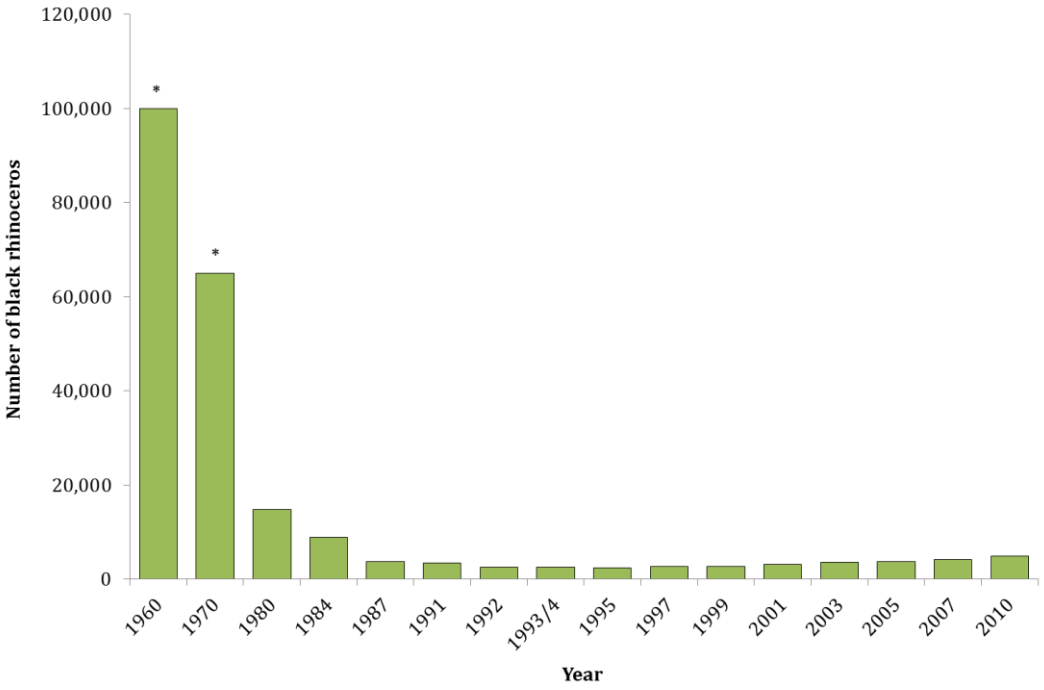


Figure 1.4: Estimated population decline in black rhinoceros (*Diceros bicornis*) since 1960. Data from AfRSG status survey and action plan (Emslie and Brooks 1999); * denotes estimated population size.

As with numerous other species, rhinoceroses have been killed by humans for thousands of years; they have suffered from habitat loss and human encroachment, and as human expansion has led to the need of more land for agriculture, been killed as vermin and agricultural pests. They have also been killed for sport, as much sought after hunting trophies; have been hunted for their meat and a variety of other body parts, including their skin which has been used to make armour, shields and good luck charms, and their blood, urine, bones and dung have all been used in traditional medicines (Bradley Martin and Bradley Martin 1982). However, by far the greatest threat to the rhinoceros has been in the second half of the twentieth century, with demand for their horn escalating uncontrollably. Although rhino horn is made from keratin, similar to compressed hair or fingernails, the supposed properties make it one of the most expensive substances on the black market, costing around $65,000 per kilogram (Biggs et al. 2013).

The main demand for rhino horn in Asia has been for use in traditional Chinese medicine, where rhino horn has been used to treat a wide variety of illnesses including fevers, headaches, epilepsy, AIDS, jaundice, stroke, influenza, poisoning, convulsions,

typhoid, abscesses, hepatitis, leukaemia, haemorrhages, rhinitis, cerebrovascular diseases, and external burns (Emslie and Brooks 1999; Toon and Toon 2002), despite there being no scientific evidence for these claims. The other main demand for rhino horn comes from Yemen and Oman, where it is used to create elaborate ceremonial daggers, called Jambiyas. Rhino horn has a translucent amber glow when held up to the light, and with age develops a unique patina, or sayfani (Emslie and Brooks 1999) which makes these daggers an important status symbol. The larger size of African rhino horn makes them more desirable for crafting Jambiyas, and the massive increase in demand during the 1970s had a catastrophic effect on African rhino populations.

Since 1995, conservation efforts and increased security have resulted in an increase in the wild population, reaching 4,880 continentally by the end of 2010 (KWS 2012), an increase of 102% in 15 years (Emslie and Knight 2011). However, remaining wild populations of black rhinoceros are highly fragmented, and many are enclosed within fenced reserves. *In-situ* conservation therefore not only requires the on-going protection of black rhinos, but biological management of the metapopulation to enhance population growth and genetic diversity. Active management such as on-going monitoring of demographic and reproductive parameters, estimating ecological carrying capacity and management of populations for maximum productivity is a necessary part of black rhino conservation. Translocation is required to move rhinos to safer areas, for genetic management, to promote breeding and to re-establish populations in areas where rhinos have become locally extinct (Emslie and Brooks 1999). However, a resurgence in poaching in recent years has threatened the recovery of the species, with the highest rates of poaching for the last 15 years, including 53 black rhinos killed in Kenya between 2007 and 2011 (Emslie 2008).

1.4.3. Rhinoceros reproduction

Much of what we know about black rhinoceros reproduction stems from *in situ* studies in the 1960's, '70's and '80's (Goddard 1967, 1970; Hall-Martin 1986; Hitchins and Anderson 1983; Schenkel and Schenkel-Hulliger 1969). From these observational studies, both age at sexual maturity (3½ - 4 years (Schenkel and Schenkel-Hulliger 1969); 3 years 8 months (Goddard 1970); 6.3 years (Hall-Martin 1986); 7- 8 years (Hitchins and Anderson 1983)), and age at first reproduction (4.75-5.25 years (Schenkel and Schenkel-Hulliger 1969); 5 years 7 months (Goddard 1970); 6.25 years

when adequate males were present or 8.9 years when only a single male was present (Hall-Martin 1986); 12 years (Hitchins and Anderson 1983)) were estimated based on either observation of sexual behaviour or based on subtraction from dates of parturition. In captivity, early published reports based on behavioural observations indicated a mean age at sexual maturity of 5.25 years (range 3-10 years), and average age at first birth of 8.8 years (Smith and Read 1992). However, reports from individual zoos suggested conception could occur earlier, with a female at Hiroshima conceiving at 3.75 years (Smith and Read 1992).

Observational data for age at sexual maturity for males was more difficult to obtain, as males are often not able to maintain a territory until 8-10 years, and so may not have access to receptive females until this time (Schenkel and Schenkel-Hulliger 1969). However, based on histological investigation of testes (Hitchins and Anderson 1983) spermatogenesis may commence between the ages of 7 and 8 years, and individual reports exist of males mating as early as 4 years 5 months (Goddard 1970), 4 years 6 months (Greed 1967), or 7 years of age (Dittrich 1967).

Black rhinos are polyoestrous (Hitchins and Anderson 1983), meaning a non-pregnant female has the potential to come into oestrus multiple times over the course of a year. There has been some evidence for seasonality *in situ* (Hitchins and Anderson 1983), but not *ex situ* (Roth 2006). Reports of oestrous cycle length and duration of oestrus obtained from observations of sexual behaviour indicated that a bull may be in attendance for 6-7 days, but the female was only receptive for a single day, with mean oestrus cycle length recorded as 35 days (normal range 26-46 days) (Hitchins and Anderson 1983). Captive reports based on behavioural signs and attempted matings suggest oestrous cycles of between 18 days (Hallstrom 1967), 21-45 days (Greed 1967), 26-30 days (Dittrich 1967), 28-30 days (Yamamoto 1967), and 30-35 days (Gowda 1967). Estimates of gestation length were based on observations of mating behaviour to parturition, and early reports varied from 450-545 days (15-18 months) (Goddard 1967; Ritchie 1963). However, although variation in length does exist, such as 419-476 days (Jarvis 1967), current estimates agree on a gestation length of 15-16 months in this species (Roth 2006) (Table 1.1).

Black rhinoceros females may lactate for at least 18 months (Gregory et al. 1965), but do not appear to exhibit lactational anoestrous. One report from a captive female at Hannover zoo was observed to be in oestrus 20 days following giving birth (Dittrich 1967), and the same female was reported as cycling regularly every 25-30 days

(Goddard 1967) until conception approximately 12 months after having given birth. Other captive females have been reported as resuming cyclicity 7 months post-partum (Yamamoto 1967). *In situ*, Goddard (1967) also reported a female that lactated for 17.5 months, but had resumed cyclicity 3 months after parturition. Perhaps rhinoceros ovarian activity resembles that of the horse, where the ovary is not suppressed by lactation and the female continues to cycle regularly after the first post-partum oestrus (King 1965). This lack of prolonged lactational anoestrous has subsequently been confirmed with hormone analysis (Brown et al. 2001), where females generally resumed cyclicity within 3-10 months post-partum. However, despite this apparent resumption of cyclicity following parturition, inter birth intervals can also be quite variable between females, but have been reported as 27 months (Goddard 1967), 26 months (Joubert and Eloff 1971), and 32 months (Hall-Martin 1986). The shortest interval reported in captivity is 16 months (Smith and Read 1992), indicating potential conception during first post-partum oestrus. However, this is relatively rare, and generally calving intervals of 2-2 ½ years are considered to be ideal (Smith and Read 1992).

In recent years the use of hormone analysis has enabled the additional measure of physiological state, either using serum (Berkeley et al. 1997), urine (Brett et al. 1989; Hindle et al. 1992; Hodges and Green 1989; Ramsay et al. 1987), faeces (Berkeley et al. 1997; Brown et al. 2001; Garnier et al. 2002; Lance et al. 2001; Schwarzenberger et al. 1993; Schwarzenberger et al. 1996b) or saliva (Czekala and Callison 1996) to measure reproductive hormones. These techniques have not only proven useful for determining oestrous cycle length *in situ* (Garnier et al. 2002), but can also be used for pregnancy diagnosis in the field (MacDonald et al. 2008). Furthermore, research on *ex situ* black rhinos has taught us a great deal about the reproductive physiology of this species, through the use of longitudinal studies of known individuals, and the use of ultrasound (Radcliffe et al. 2001) to observe the ovarian and foetal changes that occur during the oestrous cycle and pregnancy.

Although most studies had agreed on average oestrous cycles of around 26 days (Table 1.1), based on endocrine data, there is still much that we do not know about black rhinoceros reproduction, including the occurrence of erratic oestrous cyclicity. Erratic patterns of oestrous cyclicity have been reported, with cycles shorter than 20 days, and longer than 32 days also being reported in a longitudinal survey on black rhinoceros in America (Brown et al. 2001). There has also been some indication of variable oestrous cycle length *in situ* from behavioural observations of oestrus, where one female was

observed in oestrus every 67-84 days prior to her first calf, only cycling more regularly every 21 days following her first calf (Hitchins and Anderson 1983). Garnier et al (2002) also reported that although three-quarters of oestrous cycles observed had a total duration (mean ± s.e.m) of 26.8+/- 1 days, extended cycles lasting approximately twice as long were also observed.

The existence of two distinct cycle lengths has also been observed across multiple studies in captive white rhinoceros (*Ceratotherium simum*); with both 30-35 day cycles (Brown et al. 2001; Hindle et al. 1992; Patton et al. 1999; Radcliffe et al. 1997) and 65-70 day cycles (Brown et al. 2001; Patton et al. 1999; Schwarzenberger et al. 1998) being described, and may both occur within an individual over a number of months (Patton et al. 1999). However, the reason behind these two cycle lengths is not yet understood (Roth 2006), although as yet, only the shorter type has been observed to be fertile (Brown et al. 2001; Schwarzenberger et al. 1998). Furthermore in this species, prolonged periods of acyclicity are also apparent, all of which have been a recognised factor leading to poor reproductive output in captivity (Brown et al. 2001; Patton et al. 1999; Roth 2006; Schwarzenberger et al. 1998).

A number of hypotheses have been proposed for the occurrence of irregular cyclicity in the white rhinoceros, including premature reproductive ageing, as long periods without reproducing in captivity could lead to oocyte depletion and development of pathologies (Hermes et al. 2004). Another possibility is the role of adrenal activity, as acyclic females exhibit more variable faecal glucocorticoid concentration than cycling females (Carlstead and Brown 2005). However, other possibilities indicate a social mechanism, as females introduced to novel males have resumed cyclicity (Patton et al. 1999), and the potential influence of the captive diet (Berkeley et al. 2011; Patisaul 2012; Tubbs et al. 2012). Furthermore, social influences may also be involved in this species, particularly in the F1 generation that exhibit particular poor reproductive success in captivity (Swaisgood et al. 2006). However, the presence of irregular cyclicity in the black rhinoceros has to date been far less studied, but could also be a contributing factor to the inconsistent rates of reproduction reported for this species in captivity (Smith and Read 1992).

Reproductive endocrinology of males has been less common, with to date, testosterone concentration being determined in only one study *in situ* (Brett et al. 1989), and two studies *ex situ* (Brown et al. 2001; Christensen et al. 2009). Faecal testosterone metabolites remain relatively constant across the seasons (Brown et al. 2001), but

early indications suggest that there may be differences in males according to their social environment (Christensen et al. 2009), with the presence of both males and females perhaps stimulating testosterone production.

1.4.4. Rhinoceros in captivity

Rhinoceros have been held under human care for over 2000 years (Rookmaaker 1998). Currently, four of the five species of rhinoceros are currently kept in captivity, including two of the three subspecies of black rhinoceros (*Diceros bicornis michaeli* and *D. b. minor*), both subspecies of white rhinoceros (*Ceratotherium simum simum* and *C. s. cottoni*), the Indian rhinoceros (*Rhinoceros unicornis*) and one of the three subspecies of Sumatran rhinoceros (*Dicerorhinus sumatrensis*). These populations play a vital role in the conservation of these species; not only are they involved in captive breeding programs in an attempt to maintain viable populations as a vital reserve for endangered wild populations, but they also play a crucial role in raising awareness to these species' plight, and act as a potential source for re-introduction (Candra et al. 2008; Emslie 2012a; Fyumagwa and Nyahongo 2010; Holečková 2010).

However, captive populations of rhinoceros are generally not self-sustaining (Roth 2006), with sub-optimal reproduction and high mortality in the black rhinoceros (Smith and Read 1992), poor F1 reproduction in white rhinoceros (Swaisgood et al. 2006), and Sumatran rhinoceros (Roth 2006), and the Indian rhinoceros suffering from a lack of genetic diversity (Roth 2006). Careful management is therefore required to maximise the conservation potential of these *ex situ* populations, and understand factors that may influence population performance. The focus of this thesis is the European captive population of black rhinoceros, which as yet has not been the focus of research investigating population viability, but which could benefit from a collaborative multi-institutional approach to improving population performance (M. Pilgrim, *pers. comm.*). As of 31st December 2012, there were 82 black rhinos in Europe, housed at 17 institutions. Of these, all but two were of the eastern subspecies, *D. b. michaeli*, which will be the focus of this study.

Table 1.1: Reproductive parameters including oestrous cycle length, gestation and the resumption of cyclicity post-partum for the black rhinoceros, based on endocrine data using a number of sample media from published studies.

Location	Sample media	Oestrous cycle length (days ±s.e.m)	range	Irregular cyclicity observed	Gestation length	Resumption of oestrous post-partum	Reference
Ex situ	Serum/Faeces	26			468		(Berkeley et al. 1997)
Ex situ	Faeces	26.8 ±0.5	14-60	<20, >32, acyclic		3-10 months	(Brown et al. 2001)
In situ	Faeces				450-456		(Garnier et al. 1998)
In situ	Faeces	26.8 ±1	19-48	IIa ≥40d, ++ luteal IIb ≥40d, ++ follicular		4-4.5 months	(Garnier et al. 2002)
Ex situ	Urine	21/22					(Hindle et al. 1992)
Ex situ	Ultrasound	26 ±1.4		Persistent structure up to 30 days			(Radcliffe et al. 2001)
	Faeces				465-475		
Ex situ	Faeces	24-26.5			440-470	3 months	(Schwarzenberger et al. 1993)

1.5. Aims of this thesis

With fewer than 5000 black rhinoceros remaining across continental Africa and the threat of poaching on-going, *ex situ* populations play a vital role in the conservation of this species. However, for *ex situ* populations to fulfil their role, it is important that they are self-sustaining, both demographically and genetically, to maximise their future viability. However, as is the case with many other captive breeding programmes (Leus et al. 2011a; Long et al. 2011), *ex situ* populations of black rhinoceros in North America have not previously been self-sustaining (Carlstead and Brown 2005; Carlstead et al. 1999a; Carlstead et al. 1999b; Smith and Read 1992), as high rates of mortality and inconsistent rates of reproduction have prevented population growth. However, the cause of sub-optimal performance is not yet understood, and the performance of the European population of eastern black rhinoceros has yet to be formally assessed. The aim of this thesis was therefore to determine the sustainability of European captive population of eastern black rhinoceros, and investigate factors that may influence population performance, to maximise its conservation potential.

The first aim of this thesis was to gain a better understanding of factors that may influence population performance in this species, to determine biological limits for key demographic parameters relating to fecundity, mortality and population structure, and identify where there may be potential to improve population performance. In Chapter 3 demographic data compiled on *in situ* populations of eastern black rhinoceros in eight Kenyan reserves were used to ascertain how this species performs under natural conditions. This allowed a comparison of growth rates between populations, and the estimation of several indicators of population performance relating to mortality, reproduction and population structure. Through a better understanding of the normal range and variability both between individuals and across reserves, this information could then also be used as a reference, by which to guide the next chapter of this thesis.

The second aim of this thesis was to determine the viability of the European captive population of eastern black rhinoceros, and investigate ways in which population performance could be improved. In Chapter 4, historical demographic data from the European endangered species breeding programme (EEP) studbook were used to calculate demographic parameters for this population. These were then used to estimate the demographic sustainability of the population through simulated population projection, and through comparison of performance indicators to those calculated in Chapter 3, potential areas for improvement could be determined.

Furthermore, the genetic viability of the population was also assessed using known pedigrees to determine historical founder representation within the current population.

The third aim of this thesis was to investigate reproductive hormones within breeding and non-breeding males and females within this population, to determine whether intrinsic differences may be related to differential reproductive success. The necessary endocrine methods were developed and validated for use in Chapter 2, allowing the use of non-invasive faecal sampling to investigate reproductive and adrenal hormones in this species. In Chapter 5, faecal samples collected from 23 male and 39 female eastern black rhinos at 13 institutions across Europe, representing 90% of the population, were analysed for either testosterone or progesterone metabolite concentration. In females, progesterone metabolites were used to characterise oestrous cyclicity, based on previously established techniques. Mature rhinos were separated into categories according to whether they had previously produced a living calf, and whether they had produced a calf during the last seven year period, to determine whether any differences in reproductive hormones were related to prior reproductive success.

The fourth aim of this thesis was perform an exploratory analysis to investigate whether extrinsic factors relating to the captive environment may be related to adrenal activity, or to differences in reproductive success. To achieve this, the same faecal samples as used in Chapter 5 were used in Chapter 6, and analysed for glucocorticoid metabolite concentration, in both males and females. Information was also gathered on the social and physical environment and on behavioural characteristics of each individual, to determine whether either environmental factors, or how individuals respond to their environment, may influence either adrenal activity or reproductive success. Furthermore, this same information was also analysed with respect to male testosterone metabolite concentration, to investigate whether any differences in testosterone between individuals might be related to extrinsic factors relating to the captive environment.

The final aim of this thesis was to investigate reproductive cyclicity within females, using a longitudinal approach to investigate whether any differences in hormone metabolite concentration were apparent between different cycle types. The occurrence of erratic oestrous cyclicity and irregular cycle lengths have previously been described in this species (Brown et al. 2001; Garnier et al. 2002), but the potential causes and

consequences of these different cycle types has yet to be established. In Chapter 7, faecal samples collected every other day from 18 females within this population, over a period of 9-15 months, were used to investigate changes in hormone metabolite concentrations over time. In particular, hormone concentrations were compared between different cycle types, and during the preceding period, to determine whether differences in current or prior exposure to hormones may influence the occurrence of irregular cyclicity. Furthermore, any potential differences between females that had previously produced offspring, and those that had not were investigated.

Finally, in Chapter 8 of this thesis, the findings of this research are summarised, and the implications for future population management discussed. Potential avenues for further investigation are also suggested, to better understand reproduction and maximise population performance in this species.

CHAPTER 2

2. MEASURING REPRODUCTIVE AND ADRENAL HORMONES IN THE BLACK RHINOCEROS: METHOD DEVELOPMENT AND VALIDATION.

Summary

Endocrinology can be a useful tool to help understand the physiology behind processes such as reproduction, response to potential stressors, and behaviour. However in free-ranging or endangered species, or where longitudinal sampling is important, collecting blood samples to measure circulating hormones can be problematic, so a non-invasive method of sample collection can be beneficial.

In order to investigate reproductive and adrenal hormones in the European captive population of eastern black rhinoceros, methods to utilise non-invasive faecal hormone analysis were required. Prior to embarking on this study, a number of previously established enzyme immunoassays (EIAs) designed to measure progesterone (CL425; C.J. Munro), oestradiol (R4972; C.J. Munro), testosterone (R156/7; C.J. Munro) and corticosterone (CJM006; C.J. Munro) were first validated, to ensure their appropriate use in this species. Furthermore, a number of modifications were necessary to improve the reliability of data obtained, by minimising environmental influences. Once protocols were established, standard validation techniques were conducted, including parallelism, matrix interference assessment and high-performance liquid chromatography. This also allowed confirmation that a potential confound of cross-reactivity between assays, as highlighted by previous studies, was not an issue with the EIAs used for this study. Finally, biological validation was conducted opportunistically, to confirm that hormone metabolites present in faeces were indeed representative of the biological response within the body.

Following all of these validation procedures, these enzyme immunoassays could confidently be used to investigate reproductive and adrenal hormones in the black rhinoceros.

2.1. Introduction

The endocrine system is one of the main control systems within the body which, along with the nervous system, monitor the internal and external environment and make any necessary adaptive changes. The endocrine system acts through the production of hormones; chemical substances produced by specialised cells, which act as messengers to communicate with target cells in response to a stimulus. This communication can occur on different levels; intracrine cells produce hormones which act within the same cell; autocrine cells secrete hormones into the intracellular space which then act upon the same cell; paracrine cells transmit their messages to neighbouring cells within a tissue; whereas endocrine cells produce hormones to transmit signals through the circulatory system to target cells around the body. Endocrine glands may also communicate with the central nervous system, known as the neuroendocrine system, enabling the production of hormones in response to external stimuli. Hormones are involved in many processes within the body, including growth and development, metabolism, reproduction, maintenance of homeostasis, and responses to changes in the environment.

As hormones are carried in the circulatory system, measuring their presence or absence allows us to monitor internal physiology. For example, reproductive hormones can be used to determine basic reproductive parameters, such as seasonality (Walker et al. 2002), reproductive cyclicity and pregnancy diagnosis (Schwarzenberger et al. 1996a); to understand reproductive behaviour (Ganswindt et al. 2003), or to allow the discrimination between pregnancy and pseudopregnancy (Dehnhard et al. 2012). Measuring glucocorticoids allows us to investigate how individuals respond to a variety of potential challenges in their environment, such as the impact of anthropogenic disturbance (Arlettaz et al. 2007; Creel et al. 2002), habitat change (Ben Cash and Holberton 2005; Martinez-Mota et al. 2004), population density (Elsey et al. 1990; Nicholson et al. 2009), social interactions (Edwards et al. 2013), environmental conditions (Boinski et al. 1999; Moreira et al. 2007), and translocation (Viijoen et al. 2008). Furthermore, hormones play a role in orchestrating behaviour, and can be useful in understanding social bonds, parental care, aggression and reproductive behaviours (Anestis 2010; Whitten et al. 1998).

However, measuring hormones in blood can have certain limitations, not least the difficulty in obtaining samples from free-ranging subjects, and the potential stress of sample collection (Wielebnowski and Watters 2007). A number of alternative sample

media can be used however, including faeces, urine, saliva, hair and feathers. Faeces and urine in particular can be beneficial as they can be completely non-invasive, and provide an integrated measure of physiological response over a number of hours, as opposed to blood measures that can be pulsatile and affected by diurnal rhythms (Touma and Palme 2005). However, hormones measured in excreta have been processed by the body, meaning hormone metabolites and conjugates are generally present in much higher quantities than the native hormones present in the circulatory system. The precise metabolites present in faeces and urine will vary between species (Palme et al. 2005), and even between sexes of the same species (Touma et al. 2003).

Hormones and their metabolites can often be measured in biological substances using immunoassays that incorporate antibodies raised against the hormone of interest. Antibodies are produced by immunising an animal with the antigen, before collecting and purifying the immune serum (Vaitukaitis et al. 1971). A normal immune response to an antigen includes multiple B-lymphocytes, each targeting a specific recognition site (known as an epitope), on the antigen (Lipman et al. 2005). As a result, a number of different antibodies are produced, with slightly different specificity and affinity; known as polyclonal antibodies. Polyclonal antibodies are relatively quick and inexpensive to produce, and often offer a higher response due to the number of epitopes recognised, but are limited to the lifetime of the animal used to produce the antiserum (Lipman et al. 2005).Alternatively, monoclonal antibodies are produced by fusing immortal myeloma cells with a single B-lymphocyte, which is specific to a single epitope on the antigen (Köhler and Milstein 1975). The resulting 'hybridoma' takes on the characteristics of both cell types; so that a single cell-line can be stored frozen under liquid nitrogen and cloned to produce the same highly-specific antibody. This has the benefit that production can be potentially unlimited, and removes the variation of antibody production between batches (Lipman et al. 2005).

Immunoassays work by establishing a competition between a synthetic labelled antigen, and the unlabelled endogenous antigen in the sample. These two components compete for access to a specific antibody, raised against the hormone of interest. The concentration of this antibody is limited, to ensure that the higher the concentration of endogenous antigen, the greater the competition with the labelled antigen. A calibration curve is created by adding serial dilutions of a synthetic standard to compete with the labelled antigen, and plotting the proportion of labelled antigen that is bound to the antibody. All unknown samples are compared against this curve to

quantify the concentration of hormone in the sample. There are two types of immunoassays that are often used; radio immunoassays (RIA) and enzyme immunoassays (EIA). Both allow the measurement of small quantities of hormones by utilising a competition reaction, but differ in the label used to quantify the hormone. RIA utilise radioactive labelled hormones which require special licences; whereas EIA use enzyme-conjugated hormones. EIAs have been developed that have the added benefit that they are relatively cheap and easy to use, and do not have the same problems associated with the use and disposal of radioactive material (Hodges et al. 2010).

Monoclonal and polyclonal antibodies can both be produced to be either hormone- or group-specific; designed to recognise a single hormone or metabolite of interest; or a number of different metabolites that possess a similar structure at the antibody binding site (Hodges et al. 2010). Group-specific antibodies can be advantageous for measuring excreted hormone metabolites, when the native form of the hormone as found in the circulatory system is often present in negligible amounts, and the exact identity of metabolites in a given species may be unknown. However, before an EIA can be used to measure hormone metabolites in any species, assays must be properly validated to ensure they are measuring the desired compounds accurately.

This chapter details the development of endocrine methods used for this study, including some necessary modifications to EIAs to ensure reliable results could be obtained, and validation steps carried out to ensure the suitability of use of EIAs in this species. Specifically, EIA methods were developed 1) to measure reproductive hormones progesterone and oestradiol (females) and testosterone (males), and glucocorticoids in black rhino faecal extract. These methods were then validated to ensure 2) the EIAs were accurately measuring hormone metabolites in black rhino faeces 3) there was minimal cross-reactivity between EIAs in measuring the hormones of interest, and 4) the desired metabolites were reflective of biological changes.

2.2. General EIA methods

An EIA consists of certain vital components including a specific antibody raised against the antigen of interest; a polystyrene microtitre plate to which the antibody is bound; a standard antigen of known concentration; the unknown sample to be measured; and an

enzyme-conjugated antigen with which the standard and unknown antigen compete for access to the antibody. There are also several reagents that are also necessary, including coating buffer which causes the antibody to adsorb to the plate; assay buffer in which all standards, sample dilutions and labelled antigen are diluted; substrate which reacts with the labelled antigen and produces a colour change for quantification; and wash solution which terminates each incubation step and removes any unbound compounds. There are some differences in the exact reagents utilised by different assay systems, such as the type of label and corresponding substrate, but the concept remains the same for all enzyme immunoassays.

2.2.1. Reagent development[1]

To measure reproductive and adrenal hormones in black rhino faecal extract, EIAs were used to measure progesterone (CL425), oestradiol (R4972 or R0008), testosterone (R156/7) and corticosterone (CJM006), all produced and supplied by Coralie Munro, UC Davis, California. The monoclonal antibody CL425 (Quidel Corporation, San Diego, CA) was raised in mice, against 4-pregene-11-ol-3, 20-dione (Bateman et al. 2009), and purified by C.J. Munro. The polyclonal antisera were all developed by C.J. Munro (modified from (Munro and Stabenfeldt 1984)); raised against corticosterone(Watson et al. 2013); 17β-oestradiol (Walker et al. 2002); and testosterone (Walker et al. 2002). The three polyclonal antibodies were developed in New Zealand White rabbits by C.J. Munro according to an immunization protocol described by Vaitukaitis et al. (1971). The hormone-enzyme conjugate (HRP) labels for all four EIAs were also made by C.J. Munro, using the mixed anhydride method methods described by Munro and Stabenfeldt (1984).

[1] Carried out at University of California, Davis, California, by Coralie Munro.

2.2.2. Initial assay set-up [2]

The next step in EIA development is to determine the suitable concentration of antibody for use within the assay. The lower the concentration of antibody used, the more sensitive the assay, as the greater the competition between the labelled and unlabelled antigens. However, there must also be sufficient colour-change for quantification following addition of the substrate, as it binds to the labelled antigen. It is also necessary to determine the correct concentration of the enzyme conjugated (labelled) antigen, as similarly, increasing the concentration used increases the final colour change but decreases the sensitivity of the assay. In order to determine the optimum concentration of these two components, a checkerboard titration can be used, which varies concentrations of each reagent, to find a combination that gives good assay sensitivity and suitable colour change.

When this has been established, the next step is to set up the standard calibration curve, which is run on every assay, and to which the unknown concentration is compared. A suitable top standard is determined and then serially diluted two-fold, and these are incubated with the established concentration of antibody and labelled antigen. The curve is formed by plotting the percentage of bound conjugate relative to a zero standard well (i.e. full binding by the labelled antigen), against the known concentration of standard from the serial dilution. The standard concentration is inversely proportional to the percentage binding (and depth of colour produced), and the curve is plotted with standard concentration to $\log_{10}$. Ideally the curve should have a linear and relatively steep mid-section, to give high assay sensitivity, and should cover a large range of binding with 10 standards used for the calibration curve.

[2] Carried out at Chester Zoo's Wildlife Endocrinology Laboratory by Sue Walker (corticosterone, oestradiol, progesterone and testosterone EIAs), Katie Edwards (corticosterone, oestradiol and testosterone EIAs) and Vicki Norton (testosterone EIA).

2.2.3. Faecal sample preparation and extraction

Steroid hormones are hydrophobic, and easily extracted from faecal material using a solvent such as ethanol or methanol. For this study, hormone metabolites were extracted from black rhinoceros faecal material using a shaking extraction method (see Appendix 1) and 90% methanol adapted from Walker et al. (2002). The inclusion of 10% water in the extracting solvent assists with recovery of more hydrophilic conjugated steroids that could be present in excreted material (Brown 2006; Brown 2011). In brief, 0.5 g of faecal material was weighed from each sample, and 5ml of 90% methanol added. This was then shaken overnight on an orbital shaker before being centrifuged for 20 minutes at 598g and the supernatant collected. This was then evaporated to dryness under air in a fume cupboard, before being subsequently re-suspended in 1ml 100% methanol. The faecal extract was then stored at -20°C, before being diluted as necessary for running on the enzyme immunoassay (EIA) of interest.

2.2.4. Enzyme immunoassays

2.2.4.1. Single antibody EIA

In a single antibody EIA, the antigen-specific antibody is adsorbed to the microtitre plate, before standards, controls, labelled conjugate, and unknown samples are added. Hormone metabolites were measured in black rhino faecal extract using single antibody EIAs for progesterone, corticosterone and oestradiol EIA's, after Munro and Stabenfeldt (1984), and modified from Young et al. (2004) (for detailed EIA protocols, see Appendix 1). In brief, polyclonal antibody (CL425; CJM006 or R4972; all provided by C.J. Munro, U.C. Davis; See Appendix 2 for antibody cross-reactivities) was diluted in coating buffer (0.05M $NaHCO_3$, pH 9.6), 1:10,000, 1:15,000 or 1:20,000 respectively, and 50µl added to columns 2-12 (See Appendix 1 for plate layout) of a 96-well NUNC Maxisorb microtitre plate (Thermo Fisher Scientific, UK). Plates were incubated overnight at 4°C, and were ready to use the following day (CL425 and R4972) or the subsequent day (CJM006). Once coated, plates could be used for up to 1 week. Plates coated with R4972 antibody were washed five times with wash solution (0.15M NaCl, 0.05% Tween 20), before loading with 50µl EIA buffer (0.1M $NaPO_4$, 0.149M NaCl, 0.1% bovine serum albumin, pH 7.0) and incubated at room temperature for 1-5 hours prior to use. Plates coated with CL425 or CJM006 were washed five times with wash solution immediately prior to use. Standards (P0130: 0.0156 – 4.0ng/ml; C2505: 0.078 –

20ng/ml; or E8875: 0.0975 – 25ng/ml; Sigma-Aldrich, Dorset, UK), synthetic controls (30% and 70% binding), biological control (female black rhino extract diluted 1:70; 1:20 or 1:50) or samples diluted in EIA buffer (diluted 1:70 or 1:1050 for cyclicity and pregnancy respectively; 1:20 or 1:50), were run in duplicate (CL425 and CJM006 50µl per well; R4972 20µl per well), followed by 50µl per well hormone-horseradish peroxidase conjugate (diluted 1:35,000, 1:70,000 or 1:65,000 in EIA buffer; C.J. Munro, U.C. Davis). Plates were sealed and incubated for two hours at room temperature (the standard protocol); the corticosterone EIA protocol was modified prior to use to improve reliability (see section 2.3 for details), and so plates were incubated in the dark.

Following incubation, plates were washed five times with wash solution before addition of 100µl per well substrate (0.4mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6mM H_2O_2, 0.05M citrate, pH 4.0), and incubated at room temperature (CJM006 in the dark), before reading at 405nm once optical density of 0 wells reached 0.8 to 1.0.

2.2.4.2. Double antibody EIA

In double antibody EIAs, a second (non-specific) antibody that recognises the first (antigen-specific) antibody, is used to coat the plate, and a blocking buffer is added to prevent non-specific binding. The first antibody is added along with the samples, standards, controls and labelled conjugate, which then compete to bind to the second antibody during the incubation period. This type of EIA is often used when very small amounts of hormone are present in the sample, or when problems are encountered with drift across the plate (See section 2.3.2; (Graham et al. 2001)), since the competition can only begin when the primary antibody is added in the final loading step.

Testosterone metabolites were measured in black rhino faecal extract using a double antibody EIA (for modification of EIA procedure see section 2.3; for final protocol, see Appendix 1). In brief, a non-specific immunoglobulin (goat anti-rabbit IgG R2004, Sigma-Aldrich, Dorset, UK) was diluted in coating buffer (1.0µg in 250µl per well) added to 96-well NUNC Maxisorb microtitre plate, including non-specific binding wells (NSB) (See Appendix 1 for plate layout). Plates were incubated at room temperature

overnight, before IgG was discarded and 300µl Tris blocking buffer (0.02M Trizma, 0.30M NaCl, 1.0% BSA, pH 7.5) added per well. Once blocked, plates could be used after two hours, or for up to one week. Plates were then washed five times with wash solution, before 50µl assay buffer added per well, followed by standards (T1500: 0.046 – 12ng/ml; Sigma-Aldrich, Dorset, UK), synthetic controls (30% and 70% binding), biological control (male black rhino extract diluted 1:20) or samples diluted in assay buffer (1:20), all run in duplicate (50µl per well), followed by 50µl per well hormone-horseradish peroxidase conjugate (diluted 1:45,000 in assay buffer; C.J. Munro, U.C. Davis). Finally, 50µl per well primary antibody (R156/7 diluted 1:25,000 in assay buffer; C.J. Munro, U.C. Davis; See Appendix 2 for antibody cross-reactivities) was added to all wells excluding the NSB's. Plates were sealed and incubated for two hours at room temperature in the dark.

Following incubation, plates were washed five times with wash solution before addition of 100µl per well substrate (0.4mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6mM H_2O_2, 0.05M citrate, pH 4.0), and incubated at room temperature in the dark, before reading at 405nm once optical density of 0 wells reached 0.8 to 1.0.

2.3. Improving reliability

As part of the validation process for this study, a number of inconsistencies were encountered with some of the EIAs. In order to improve the reliability of data for this study, existing protocols were modified where necessary; to ensure intra- and inter-assay coefficients of variation (CVs) were maintained within acceptable limits of 5-10% and 10-15% respectively (Kurstak 1985; Munro and Stabenfeldt 1984).

2.3.1. Light and temperature[3]

Following the standard protocol as described in section 2.2.4.1, adapted from Munro and Stabenfeldt (1984), and using the newly developed polyclonal corticosterone antibody CJM006, a high degree of optical density variability was observed across plates when the same amount of either synthetic corticosterone standard or faecal extract was added to each well. Over a period of 10.5 weeks, the concentration of the same black rhino faecal extract varied from 91 to 249 ng/g faeces, a CV of 26.8%; and to a lesser extent in synthetic corticosterone (12.8 and 13.5% for high and low binding controls respectively). This led to highly inconsistent and therefore unreliable results. The EIA, therefore, had to be modified prior to use for this study.

Under standard conditions, two aberrant patterns were observed within the observed optical density, illustrated in Figure 2.1 Type 1 patterns showed a gradual increase or decrease down each column (a-h) or across each row (1-12) within the plate; and type 2 patterns were characterized by the outside wells being noticeably higher or lower within a row or column than the inside wells. These patterns did not occur on every plate, but when present, occurred either individually or in combination. Although the amount of variability was greater when plates were loaded with faecal extract, patterns were also observed with synthetic corticosterone.

[3] The work for this section was carried out by Rebecca Watson, Katie Edwards and Sue Walker, and is adapted from Watson, R., Munro, C., Edwards, K.L., Norton, V., Brown, J.L., Walker, S.L., 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.

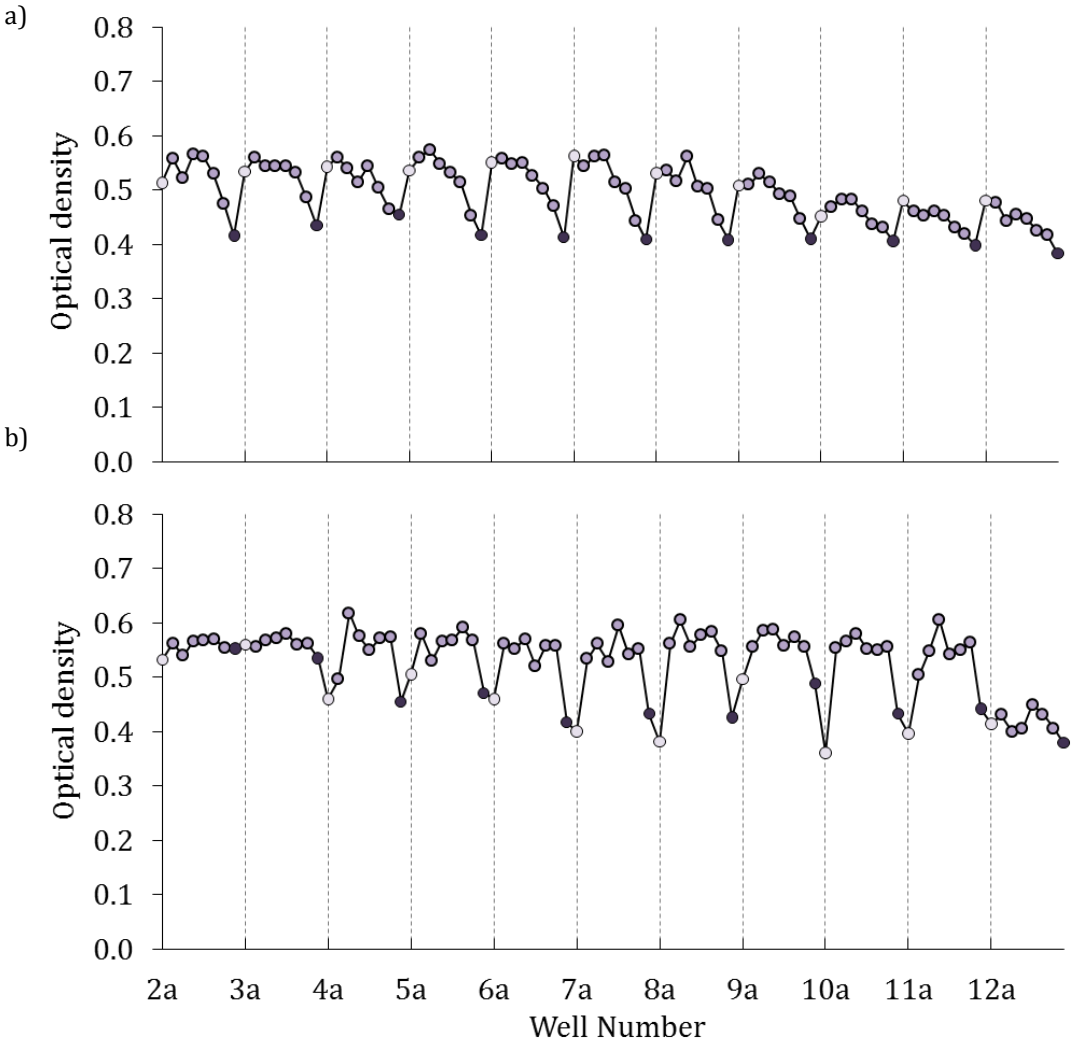


Figure 2.1: Adapted from Watson et al. (2013). Illustration of the two optical density pattern types observed prior to EIA modification. Instead of a consistent optical density between all wells, a) type 1 patterns show a gradual increase or decrease in optical density down each column (2-12) on the plate from row a (○) to row h (●); b) type 2 patterns show outer wells on the plate (typically both a (○) and h (●)) being noticeably different (higher or lower within a row) than the inner wells (◐).

A series of experiments were conducted within the laboratory ((Watson et al. 2013); see Appendix 7), to minimise the intra- and inter-assay variation. Firstly, the type of microtitre plate used was investigated, since different plate types are known to affect antibody binding (Kricka et al. 1980; Shekarchi et al. 1984), with lower affinity binding plates previously found to exhibit lower variation (Rebeski et al. 1999). The standard Nunc MaxiSorp® plates were compared with lower binding affinity Immulon IB microtitre plates. However, the occurrence of patterns in optical density were still apparent using both plate types (Figure 2.2), and were in fact increased when using the lower affinity Immulon IB plates, with patterns observed within 100% of plates, compared to only 40% of Nunc MaxiSorp® plates run in parallel.

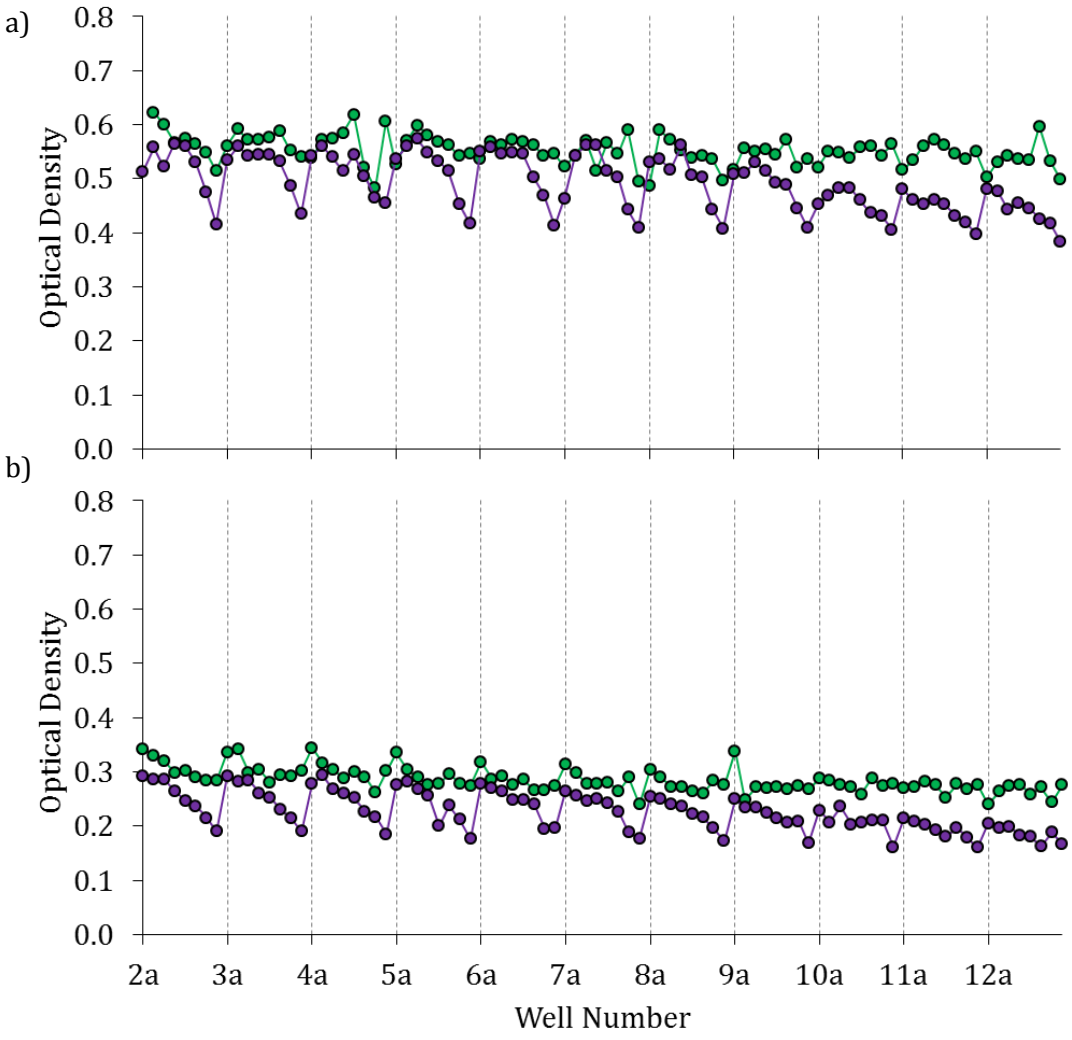


Figure 2.2: Adapted from Watson et al. (2013). Representative patterns observed on the corticosterone enzyme immunoassay run on (a) Nunc MaxiSorp® and (b) Immulon IB microtitre plates, following the addition of either synthetic corticosterone (●) or black rhino faecal extract (●).

Secondly, when excessive variation is observed within an EIA, a common solution is the addition of a secondary antibody. This addition has previously been found to reduce time-dependant drift across a plate, which is associated with the duration of plate loading, and results in differential incubation times between wells (Meyer and Hoffmann 1987). Furthermore, secondary antibody can minimise the deviation of readings from outer to inner wells caused by uneven binding of primary antibody to the well surface (Meyer and Guven 1986). The standard EIA protocol (section 2.2.4.1) was modified to include a secondary antibody coating step, use of blocking buffer to reduce non-specific binding, and the addition of the primary antibody delayed until the sample loading step (section 2.2.4.2 for full details). However, in this case, utilising a secondary antibody was beneficial only with synthetic corticosterone; it did not alleviate the patterns observed with faecal extracts (Figure 2.3).

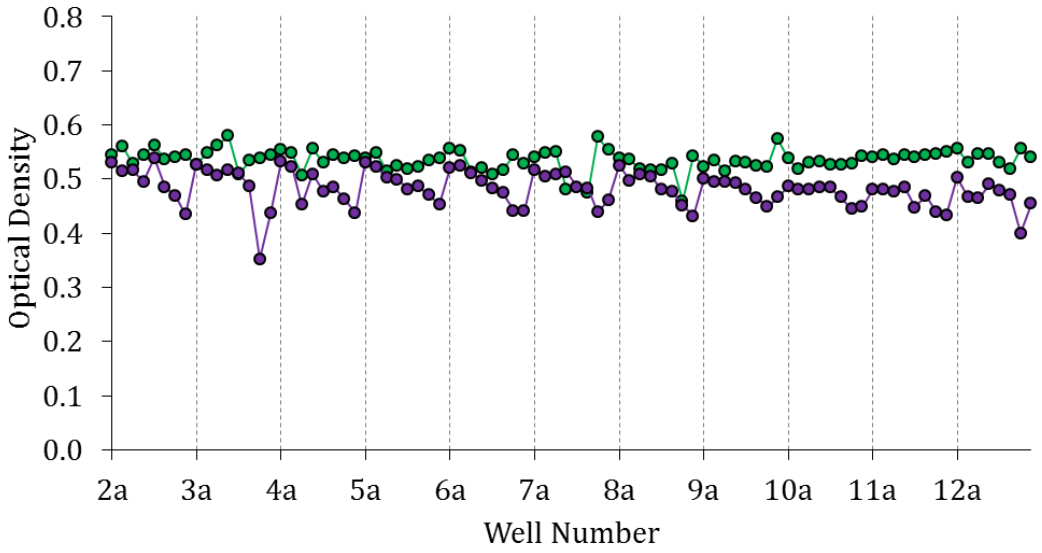


Figure 2.3: Adapted from Watson et al. (2013). Representative patterns observed on the corticosterone enzyme immunoassay run on Nunc MaxiSorp® microtitre plates coated with a secondary antibody, and run following the addition of either synthetic corticosterone (●) or black rhino faecal extract (●).

Instead, by manipulating the direction of a light source, the same patterns that had previously been apparent under standard conditions were artificially induced, with optical densities gradually decreasing closer to the light source (Figure 2.4). By modifying the EIA protocol to conduct all incubation steps in the dark, type 1 patterns were eliminated. However, variability was still higher than expected, and type 2 patterns were still apparent, suggesting that the observed patterns were not entirely due to variation in light. Temperature can be a common cause of the 'edge effect' observed in many EIAs (Gibbs 2001), which occurs when the outer wells of a plate have different optical densities to the inner wells. This may be due to outer wells being more exposed to ambient temperatures, and therefore changing temperature more quickly than inner wells, resulting in altered enzymatic activity (Burt et al. 1979; Gibbs 2001). Indeed, the type 2 patterns observed here, including the patterns observed in the dark, reflected an 'edge effect' with dramatically different optical densities only observed in the outer-most wells.

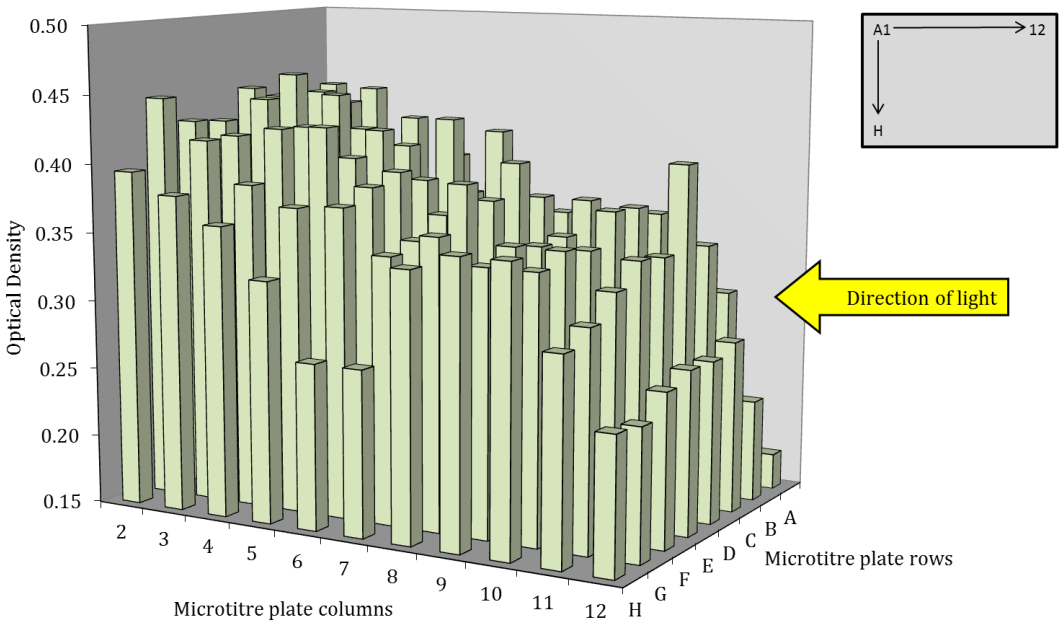


Figure 2.4: Adapted from Watson et al. (2013). Three-dimensional graph representing the variation in optical density observed between wells following the addition of black rhino faecal extract across a 96-well microtitre plate, and exposure during incubation to a natural light source from the right hand side. Each vertical column represents one well on the microtitre plate; the height of each column represents the optical density of the well.

Indeed, it is most likely that an interaction between light and temperature underlies the variation previously seen in this EIA, as ambient temperature and levels of daylight are highly variable from day to day. Prior to modification, this EIA had produced highly variable results, with the existence of two pattern types, which on different occasions may occur either alone, together or not at all. Following modification, this EIA is run using room temperature plates and reagents (as opposed to 4°C), and incubated in the dark to minimise the existence of these two pattern types (Figure 2.5), and improve the reliability of results obtained using this system.

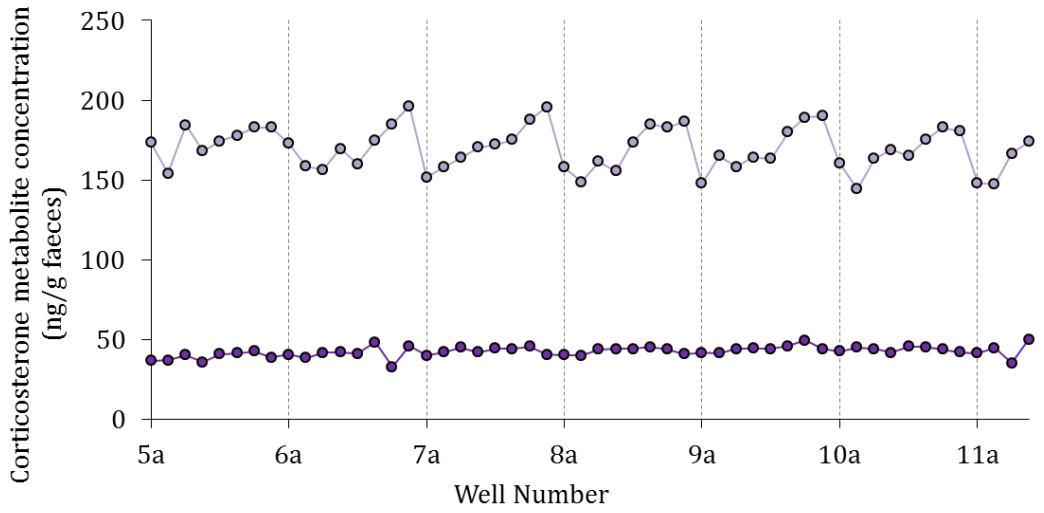


Figure 2.5: Aberrant pattern observed in corticosterone metabolite concentration (ng/g faeces) observed in black rhino faecal extract using standard protocol prior to modification (●), and pattern removed using modified protocol (●), with plates run using room temperature reagents and incubated in the dark.

2.3.2. Plate drift

Similarly, whilst validating the testosterone EIA (R156/7) for use in this study, the existence of type 1 and type 2 patterns were observed (Figure 2.6). The existing protocol was modified as with the corticosterone EIA, to include room temperature reagents and dark incubation. However, results obtained from this modified EIA were still exceeding the desired intra- and inter-assay CVs of 10% and 15% respectively. Furthermore, the variation in this case included 'plate drift', where the concentration measured increased across the plate (Figure 2.7). The problem of plate drift (Munro and Stabenfeldt 1984) is associated with the duration of plate loading, and results in differential incubation times of samples and labelled antigen between wells. The wells towards the end of the plate have a reduced incubation time to those at the start, which results in slightly reduced competition by the labelled conjugate, reduced substrate conversion and an apparent increase in sample concentration.

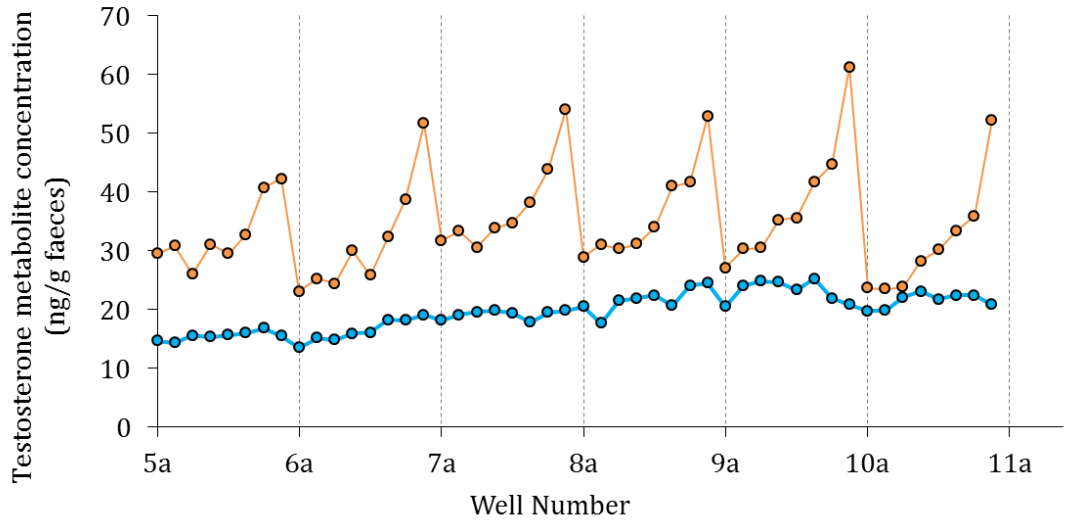


Figure 2.6: Male black rhino faecal extract run on two plates under standard conditions (full light) prior to modification, run on two separate days. An aberrant pattern was observed on day one (● CV 25.52%), but not on day two (● CV 16.45%).

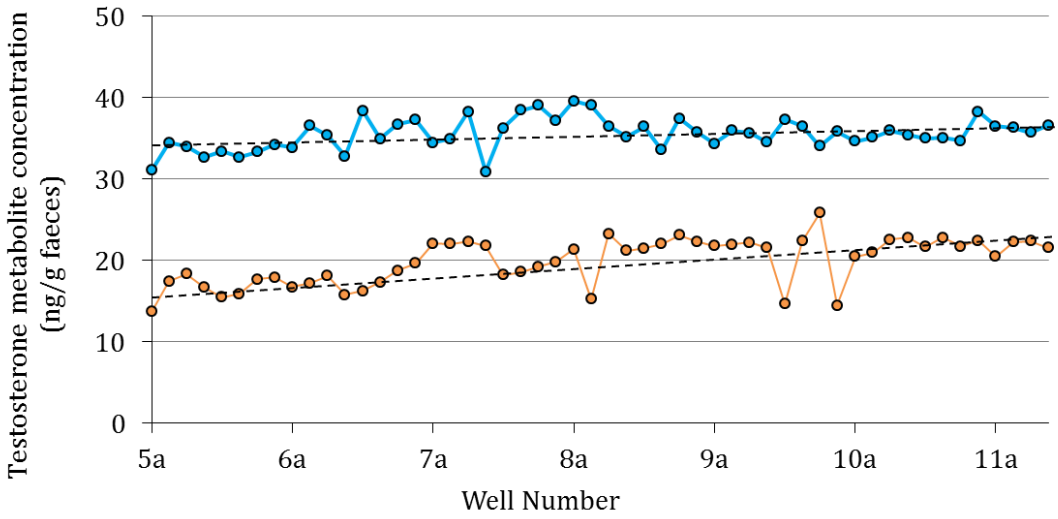


Figure 2.7: Male black rhino faecal extract run on two plates run on the same day under dark conditions, either using a single antibody, or double antibody protocol. Plate drift was more extreme on the single antibody plate (● CV 14.27%), but much reduced on the double antibody plate (● CV 5.41%).

When excessive variation is observed within an EIA, the addition of a secondary antibody has previously been found to reduce this time-dependant drift across a plate (Meyer and Hoffmann 1987). A double antibody system means that instead of using the primary (hormone-specific) antibody to bind to the microtitre plate, an antibody raised against the primary antibody is used. Samples, standards, controls, and labelled antigen are all then added before the primary antibody, which can be added almost simultaneously across the plate. The primary antibody is immobilized to the plate by being bound by the second antibody, and as the competition reaction can only begin once the primary antibody is present, drift across the plate due to differential competition is minimised (Graham et al. 2001). The standard EIA protocol (section 2.2.4.1) was modified to include a secondary antibody coating step, use of blocking buffer to reduce non-specific binding, and the addition of the primary antibody delayed until the sample loading step (section 2.2.4.2 for full details). This modification reduced intra-assay CVs from approximately 15-20% to 5-10%.

2.4. Assay suitability and species validation

Due to the wide application of EIA's with a range of sample media and numerous species, it is vital that assay suitability and performance is carefully determined and monitored. For each new species and each new hormone of interest, the enzyme immunoassay must be validated. This is to ensure that the hormone or group of metabolites of interest are present in the sample medium e.g. faeces, are present in sufficient quantities to be detected accurately, and can be measured reliably by the assay system, without any interference. There are a number of established methods for checking the suitability of an assay system for measuring a particular hormone in a particular species. Different species do not always have the same metabolites present in their faeces or urine, and the metabolites are not necessarily the same as the native hormone present in the circulatory system. Even closely related species do not necessarily have the same metabolites present, and so every new species for analysis must first go through the following validation steps. It is also important to determine that the hormones of interest are biologically relevant to the research question. For example, if measuring glucocorticoids, it is important to determine whether the substance the assay is measuring is released in response to a stressor. Biological validation is therefore necessary before any associations with behaviours or events can be made.

2.4.1. Parallelism

Performing a parallelism allows the assessment of two aspects of assay suitability. Firstly, a parallelism is used to determine whether the EIA is measuring the hormone metabolites present in a faecal extract in a similar way to the synthetic hormone to which the antibody was raised. A serial dilution of faecal extract is run in duplicate on the EIA of interest, alongside a serial dilution of the synthetic standard. Parallelism is typically assessed visually by plotting the displacement curves of the synthetic standard and the faecal extract on the EIAs. However, it is also important that the relationship be confirmed with linear regression to provide a less subjective assessment of the suitability of the EIA. A significant linear regression indicates that the percentage binding of the synthetic standard can be used to determine the percentage binding of the endogenous hormone metabolites, as binding to the antibody is proportional along the range of serial dilutions used for assessment.

Successful parallelism was obtained for female black rhino faecal extract on the progesterone, oestradiol and corticosterone EIAs, and male faecal extract on testosterone and corticosterone EIAs (Figures 2.8–2.9; Table 2.1), indicating that the metabolites in the sample are immunologically similar to the synthetic standards, and are being measured proportionately by the EIAs.

Secondly, if the EIA is indeed suitable for measuring hormone metabolites of interest, a parallelism can then be used to determine the correct dilution to run biological samples. To obtain accurate results, faecal samples should be run on the EIA at a dilution to give approximately 50% binding. This is generally the most sensitive and accurate portion of the calibration curve, being both steep and linear, so that relatively small changes in sample binding are representative of a greater difference in concentration.

2.4.2. Matrix interference assessment

Matrix interference assessment determines how accurately the EIA measures the concentration of a sample, and determines whether the sample matrix causes any interference to that measurement. A serial dilution of synthetic standard is spiked with an equal volume of a working dilution of the faecal extract, based upon the parallelism (section 2.4.1) to achieve 50% binding. Once the background concentration of the faecal extract has been accounted for (observed minus background), the observed concentration is compared to the expected concentration of the synthetic standard, using linear regression.

For all EIAs used, there was no evidence of matrix interference, as addition of diluted faecal extract to synthetic standards did not significantly alter the amount observed (Figures 2.10-2.11; Table 2.1). A high R^2 indicates that the EIA is accurately measuring the hormone metabolites, without significant interference from the matrix at the working dilution. Ideally the regression coefficient representing the gradient should be close to 1 to indicate that the faecal extract is having no influence upon the measurement of the expected concentration of synthetic standard used. However, in the case of the oestradiol EIA (R4972), the amount of immunoreactivity observed was over-estimated relative to the expected concentration, with a gradient of 3.0552. Although this EIA may be overestimating the observed concentration, a significant

regression suggests that this over-estimation is consistent across the range of concentrations used, so changes in concentration can still be inferred and this EIA can be used to investigate differences in faecal oestradiol metabolite concentration.

(a)

(b)

(c)

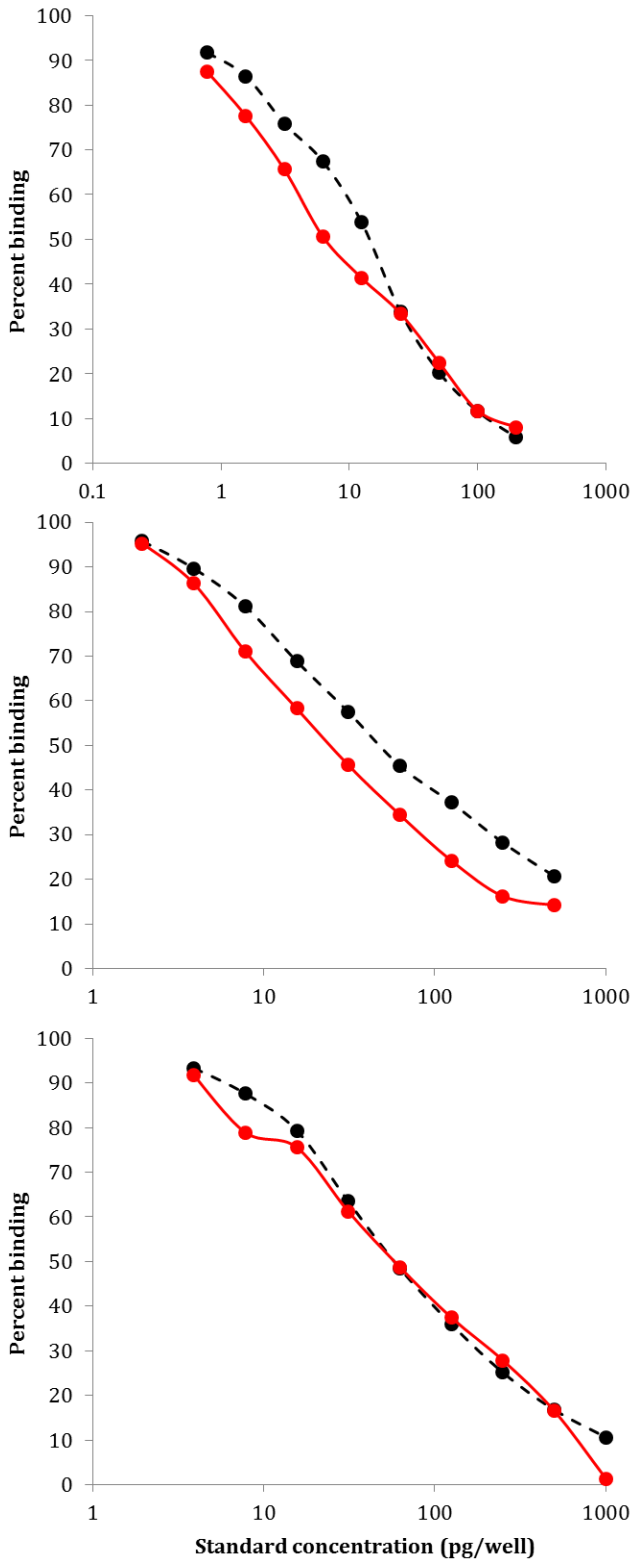
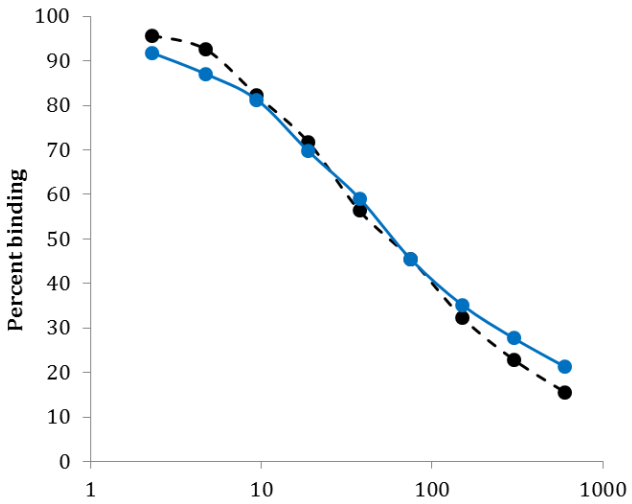


Figure 2.8: Female black rhino faecal extract demonstrates parallelism with (a) progesterone, (b) oestradiol and (c) corticosterone standard curves. Standards (●) and pooled faecal extract (●).

(a)



(b)

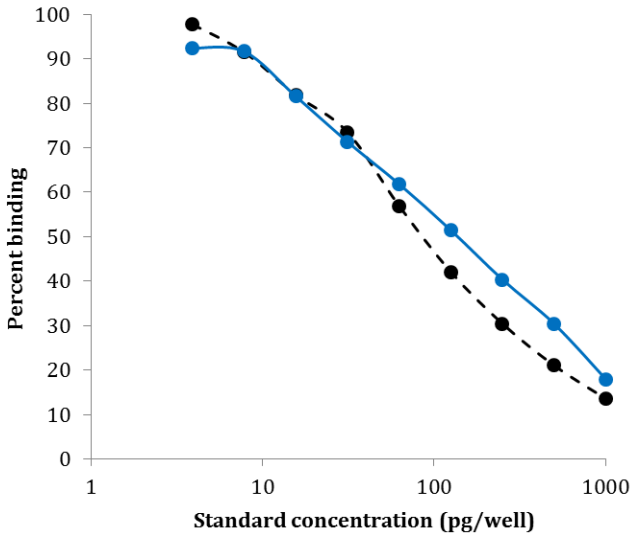


Figure 2.9: Male black rhino faecal extract demonstrates parallelism with (a) testosterone and (b) corticosterone standard curves. Standards (●) and pooled faecal extract (●).

Table 2.1: Validation of black rhino faecal extracts on multiple EIA's, through parallel displacement of faecal extract and standard curves, and matrix interference assessment.

EIA	Extract	Parallelism	Matrix interference assessment
Progesterone CL425	Female black rhino	Sample % binding = 0.851 (standard % binding) + 2.014 R^2=0.969, $F_{1,7}$=222.140, P<0.001	Observed = 0.775 (Expected) + 0.807 R^2 = 0.998, $F_{1,7}$ = 4338.484, p<0.001
Testosterone R156/7	Male black rhino	Sample % binding = 0.879 (standard % binding) + 7.298 R^2=0.997, $F_{1,7}$=2563.486, P<0.001	Observed = 1.107 (Expected) – 1.872 R^2 = 0.996 $F_{1,7}$ = 1668.608, p< 0.001
Oestradiol R4972	Female black rhino	Sample % binding = 1.094 (standard % binding) – 14.257 R^2=0.986, $F_{1,7}$=506.114, P<0.001	Observed = 3.0552 (Expected) – 10.087 R^2 = 0.983 $F_{1,5}$ = 288.582, p< 0.001
Corticosterone CJM006	Female black rhino	Sample % binding = 0.971 (standard % binding) – 0.873 R^2=0.982, $F_{1,7}$=377.007, P<0.001	Observed = 1.082 (Expected) + 2.266 R^2 = 0.999 $F_{1,7}$ = 7133.701, p< 0.001
Corticosterone CJM006	Male black rhino	Sample % binding = 0.850 (standard % binding) + 11.823 R^2=0.987, $F_{1,7}$=537.761, P<0.001	Observed = 1.013 (Expected) – 0.698 R^2 = 0.995 $F_{1,7}$ = 1471.256, p< 0.001

(a)

(b)

(c)

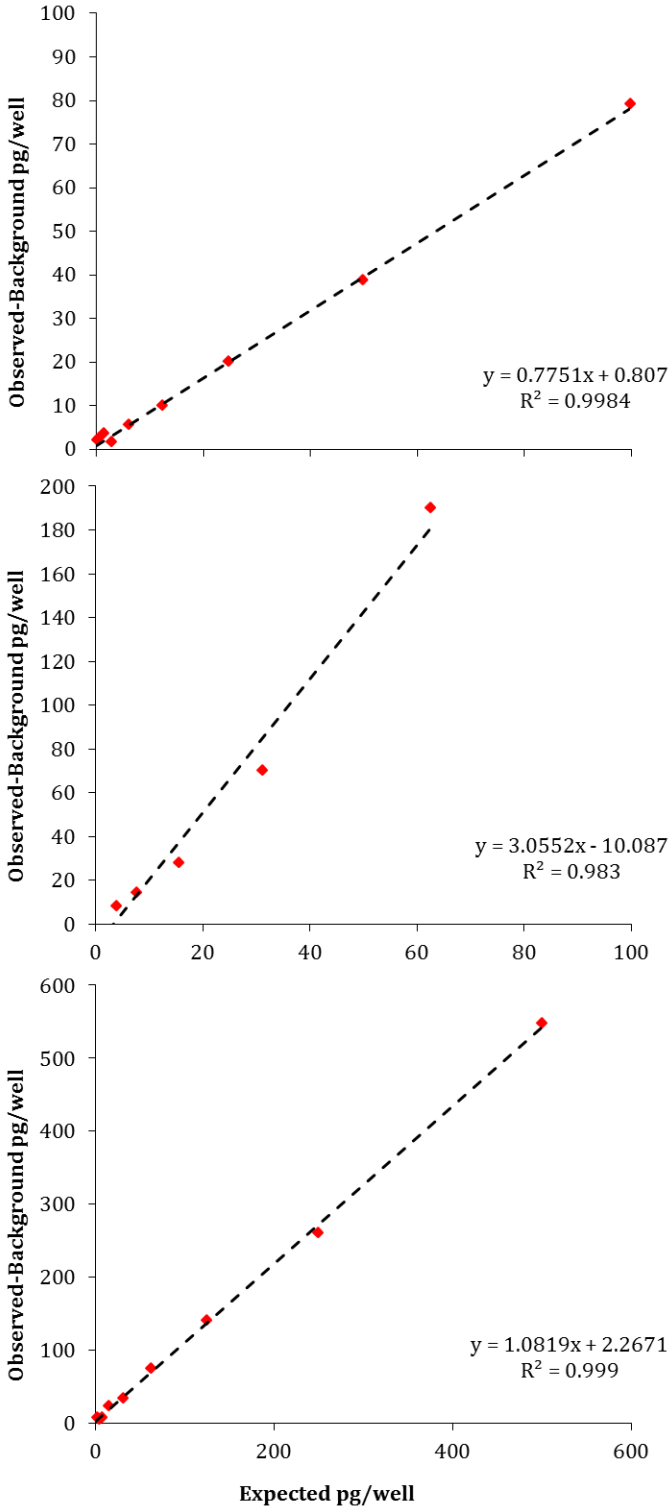
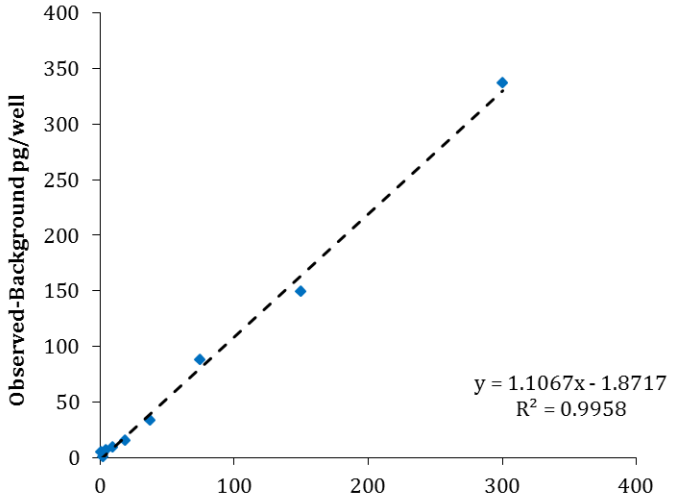


Figure 2.10: Assessment of matrix interference of female black rhino faecal extract on (a) progesterone, (b) oestradiol and (c) corticosterone standards with CL425, R4972 and CJM006 EIAs respectively.

(a)



(b)

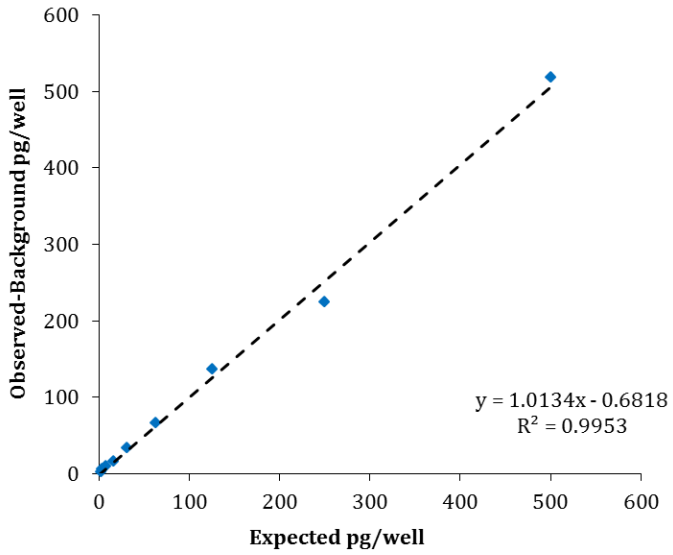


Figure 2.11: Assessment of matrix interference of male black rhino faecal extract on (a) testosterone and (b) corticosterone standards with R156/7 and CJM006 EIAs respectively.

2.4.3. High performance liquid chromatography

High performance liquid chromatography (HPLC) is a separation technique used to identify substances within a sample, based on their chemical characteristics. HPLC can be either normal- or reverse-phase, depending on the polarity of the compounds of interest; hydrophobic molecules, such as steroid hormones, can be separated by reverse-phase HPLC. Samples are first adsorbed onto a hydrophobic, non-polar stationary phase, before gradual elution with an increasing concentration of an organic solvent mobile phase. More polar compounds with lower affinity for the stationary phase are eluted off the column first, followed by less polar compounds as the concentration of solvent increases. As compounds are eluted, they are detected with a UV detector to create a chromatogram. Additionally, the eluting solvent can be separated into fractions, collected and analysed on the EIA of interest for quantification of immunoreactivity.

Faecal hormone analysis usually means measuring the metabolites of the hormone of interest which have been modified in some way to aid their excretion. As the native hormone in often not present in great quantities (Mostl and Palme 2002; Palme et al. 2005), it is therefore important to determine whether the metabolites being measured by a particular EIA are immunologically similar to the native hormone. Often the compounds that elute will not be identical to the synthetic standards used for reference, as the metabolism prior to excretion may have altered their structure and/or polarity. However, performing HPLC allows determination of the number of metabolites being detected by the EIA, and whether they have similar polarity to the standard to which the antibody was raised.

Additionally, this fractionation technique allows investigation of whether faecal metabolites are detected on multiple EIAs, exhibiting potential cross-reactivity. Since antibodies are often designed to detect a range of structurally similar metabolites, previous studies have highlighted a potential confound whereby EIAs designed to measure glucocorticoids also detect structurally similar, but biologically distinct androgens (Ganswindt et al. 2003). To investigate whether this might be an issue with the EIA used for this study, the same fractions were run on CJM006 and R156/7 EIAs, to determine whether significant immunoreactivity peaks were detected at the same elution positions, perhaps indicating that the same compounds may be being measured by the two EIA.

2.4.3.1. Sample preparation and Reverse-Phase HPLC

Male and female black rhino faecal extracts were prepared as described in section 2.2.3. Faecal extracts were evaporated to dryness, and reconstituted in 1ml 40% methanol before loading the total volume onto a pre-conditioned (4ml methanol; 4ml distilled water) C-8 cartridge (Thermo Hypersep™ C18; Thermo Fisher Scientific, Runcorn, UK). Two 0.5g extracts from the same samples were loaded onto each cartridge, to give the equivalent of 1.0g (wet-weight) faecal matter per extract. The cartridge was then washed with 5ml distilled water, before total steroids were eluted with 5 ml 100% methanol, evaporated to dryness, and reconstituted in 300µl 50% methanol. This was transferred to a microtube and centrifuged at 12,000 rpm for 5 minutes to remove any further sediment. Each filtered faecal extract (50µl aliquot) was then separated on a C-18 column (Thermo Fisher Scientific Hypersil GOLD™ 150 x 4.6mm, particle size 5µ; Runcorn, UK) with a column guard (Thermo Fisher Scientific Hypersil GOLD™ 10 x 4mm; Runcorn, UK) using a linear gradient of 20–100% methanol (MeOH; glucocorticoids and androgens) or 20-100% acetonitrile (MeCN; androgens and oestrogens) in water over 80min (1ml/min flow rate, 1ml fractions). Elution peaks were detected using a UV detector at 254nm, or using a full scan for particular standards where the chemical structure is not suited to UV detection. Additionally, reference standards known to cross react more than 0.1% with the corticosterone (CJM006), testosterone (R156/7) and oestradiol (R4972) antibodies (see Appendix 2 for cross-reactivities) were also separated using the same reverse-phase HPLC protocol. All fractions were collected, evaporated to dryness, reconstituted in 300µl assay buffer and an aliquot (50µl on CJM006 and R156/6; 20µl on R4972) assayed in duplicate for immunoreactivity on the respective EIAs.

2.4.3.2. Corticosterone EIA CJM006

Immunoreactivity profiles illustrate which fractions eluted from the HPLC contain any compounds recognised by the respective antibodies, allowing comparison of the elution positions of unknown metabolites in black rhino faecal extract with known synthetic standards.

In female faecal extract separated with a methanol mobile-phase, three main peaks of immunoreactivity were detected on the corticosterone EIA (Figure 2.12), which eluted at 37, 43 and 51 minutes respectively. These same elution positions were also detected for male faecal extract (Figure 2.13), with main peaks of immunoreactivity under methanol separation also at 37, 43 and 51 minutes. Under methanol separation, the synthetic reference standard corticosterone eluted at 37 minutes, indicating that one of the immunoreactive peaks in both male and female faecal extract was immunologically very similar to corticosterone. Another of the reference standards, desoxycorticosterone, eluted at 44 minutes, indicating that one of the other metabolites present may be similar to this compound. The third immunoreactive peak did not co-elute near any of the other reference standards used.

The male faecal extract separated using the acetonitrile mobile-phase was also analysed on the corticosterone EIA, and illustrated immunoreactive peaks at 17 and 24 minutes, co-eluting with corticosterone and desoxycorticosterone respectively.

Although in some species the metabolites present in faeces can be different (Touma et al. 2003), in black rhino faecal extract the same elution peaks were present on immunoreactivity profiles for the corticosterone EIA, indicating that the assay is measuring a number of metabolites in faecal extract, but the same appear to be present in both males and females.

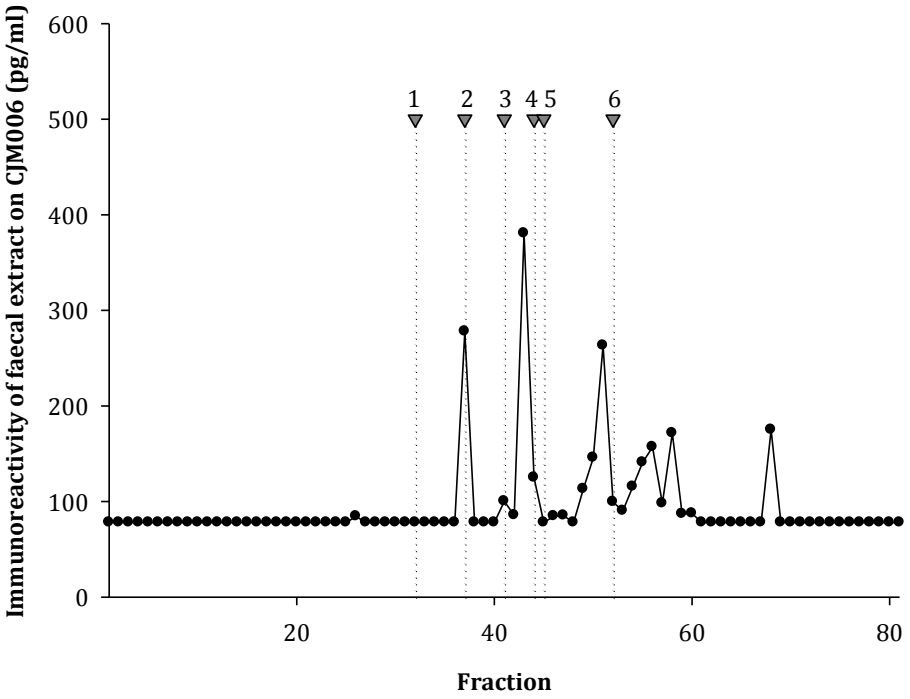
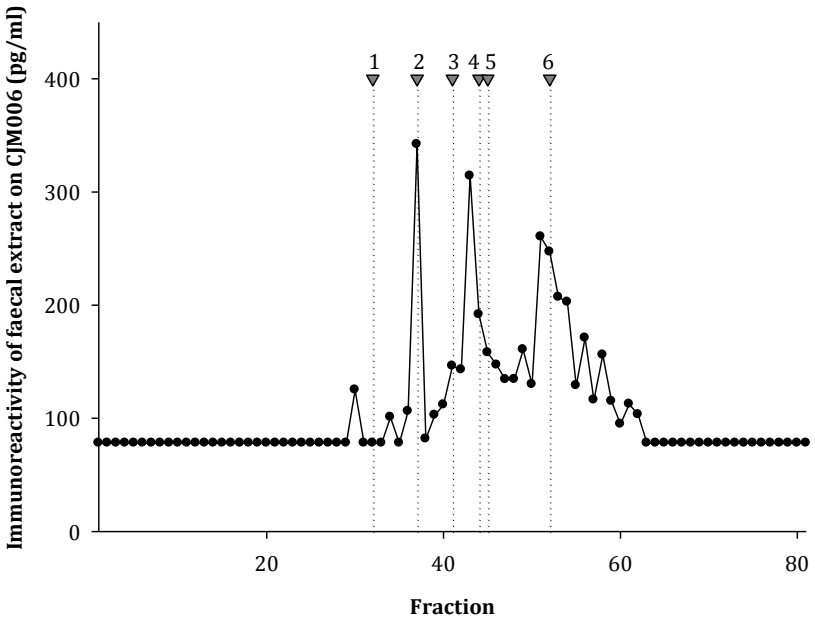


Figure 2.12: Immunoreactivity of female black rhino faecal extract, separated with methanol gradient, on corticosterone EIA CJM006. Arrows denote elution positions of synthetic standards 1) cortisol, 2) corticosterone, 3) tetrahydrocorticosterone, 4) desoxycorticosterone, 5) testosterone and 6) progesterone.

(a)



(b)

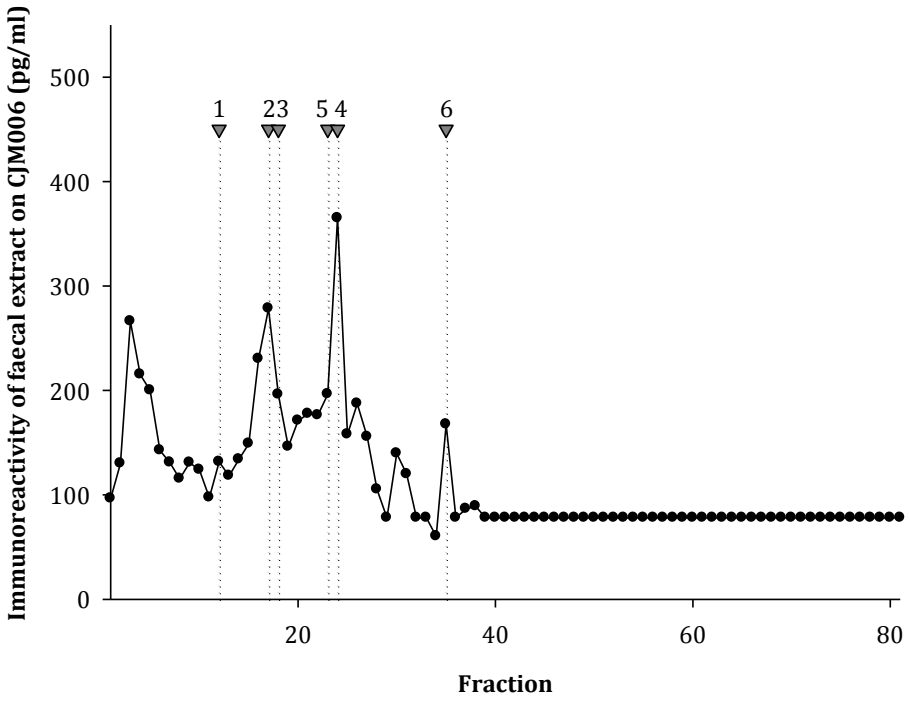


Figure 2.13: Immunoreactivity of male black rhino faecal extract, separated with (a) methanol and (b) acetonitrile gradient, on corticosterone EIA CJM006. Arrows denote elution positions of synthetic standards 1) cortisol, 2) corticosterone, 3) tetrahydrocorticosterone, 4) desoxycorticosterone, 5) testosterone and 6) progesterone.

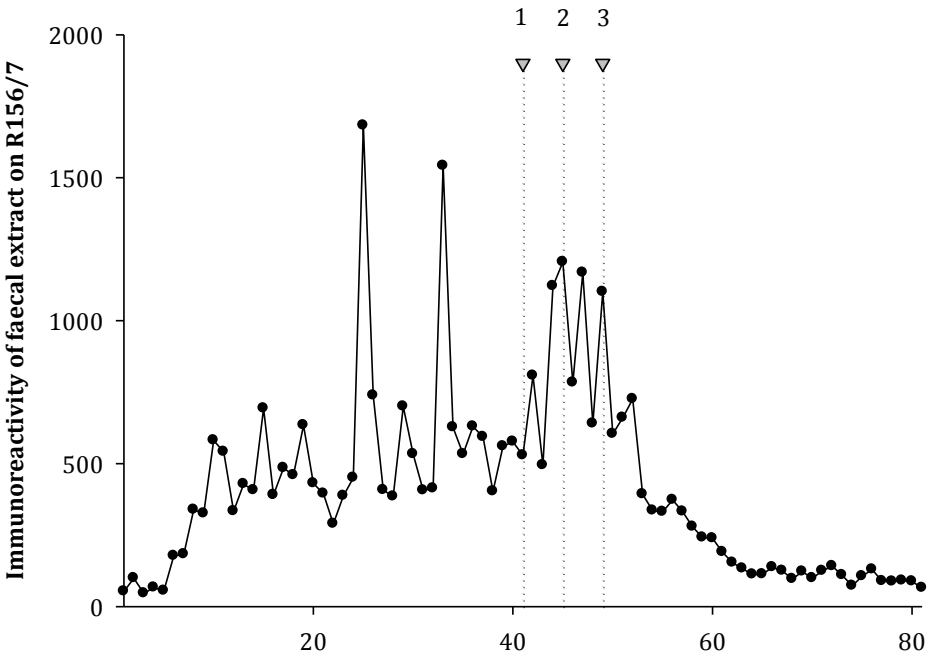
2.4.3.3. Testosterone EIA R156/7

Immunoreactivity profiles using both MeOH and MeCN separation of male black rhino faecal extract are shown in Figure 2.14, illustrating a number of peaks of immunoreactivity observed on the testosterone EIA. Using methanol separation, there were 5 clear elution peaks, three of which were in the region of the reference standards (40-50 minutes) which were known to cross-react with the R156/7 antibody. The first of these co-eluted with the reference standard testosterone at elution position 45, and the third co-eluted with 5α-dihydrotestosterone at position 49, suggesting that some of the metabolites measured by this antibody are immunologically very similar to these two compounds. The second of these three peaks eluted at position 47, but no reference standard co-eluted at this point. This peak is likely to be a compound slightly less polar than testosterone, but slightly more polar than 5α-dihydrotestosterone. The two earlier elution peaks at 25 and 33 minutes respectively could not be identified based on the reference standards used, but are more polar than all of the androgen reference standards tested on this occasion.

The acetonitrile immunoreactivity profile was slightly less clear, with a relatively large amount of immunoreactivity detected between 4-10 minutes. Other than this region, the main immunoreactivity peak eluted at 18 minutes, and could not be identified. There were also small immunoreactivity peaks at 24 and 26 minutes, around the same region as the three reference standards. However, it is felt that this separation was possibly unsuitable for detecting androgens, or alternatively, insufficient mass was put through the column to allow sufficient immunoreactivity on the EIA.

Although we cannot identify the exact metabolites being measured using this approach, when the same fractions of male faecal extract were run in parallel on the corticosterone and testosterone EIAs, no peaks of immunoreactivity were evident at the same elution positions on the two EIAs using either mobile phase (Figure 2.15). This suggests that the two EIA are not measuring significant quantities of the same compounds, indicating that cross-reactivity does not appear to be an issue with results obtained using these EIAs.

(a)



(b)

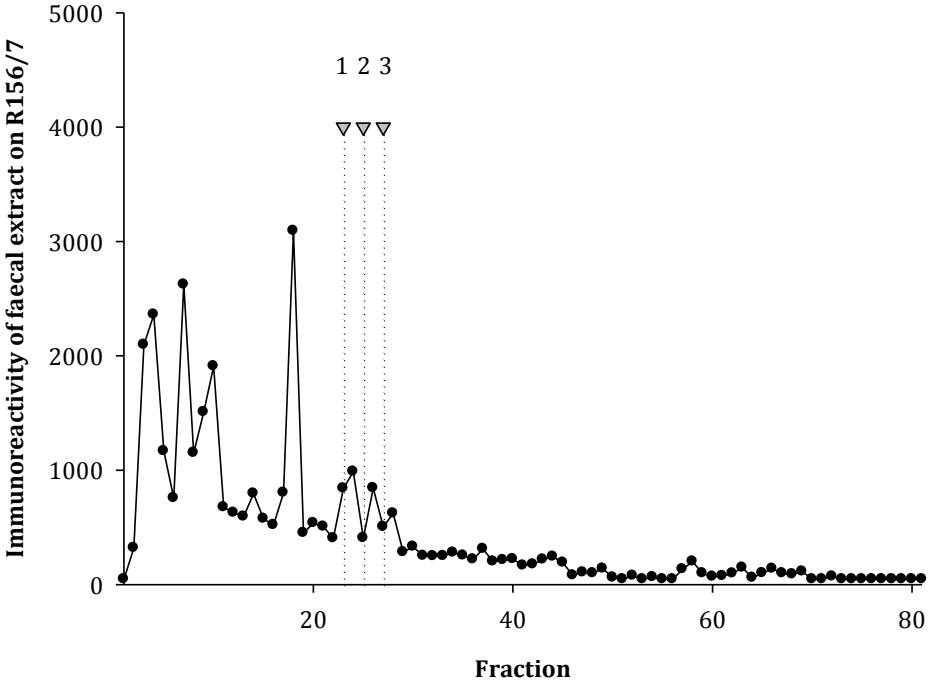
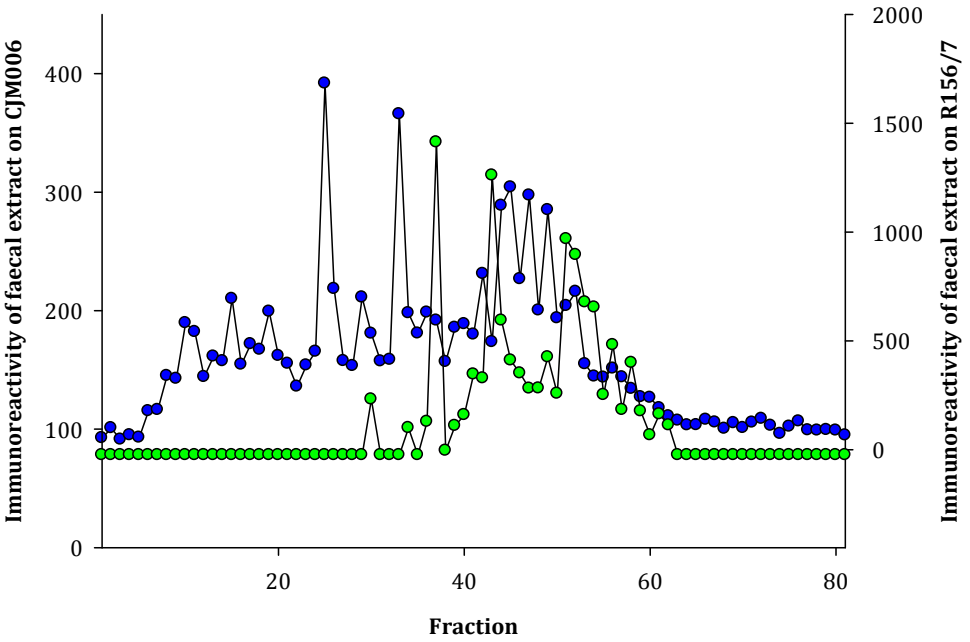


Figure 2.14: Immunoreactivity of male black rhino faecal extract, separated with (a) methanol and (b) acetonitrile gradient, on testosterone EIA R156/7. Arrows denote elution positions of synthetic standards 1) androstenedione, 2) testosterone and 3) 5α-dihydrotestosterone.

(a)

(a)



(b)

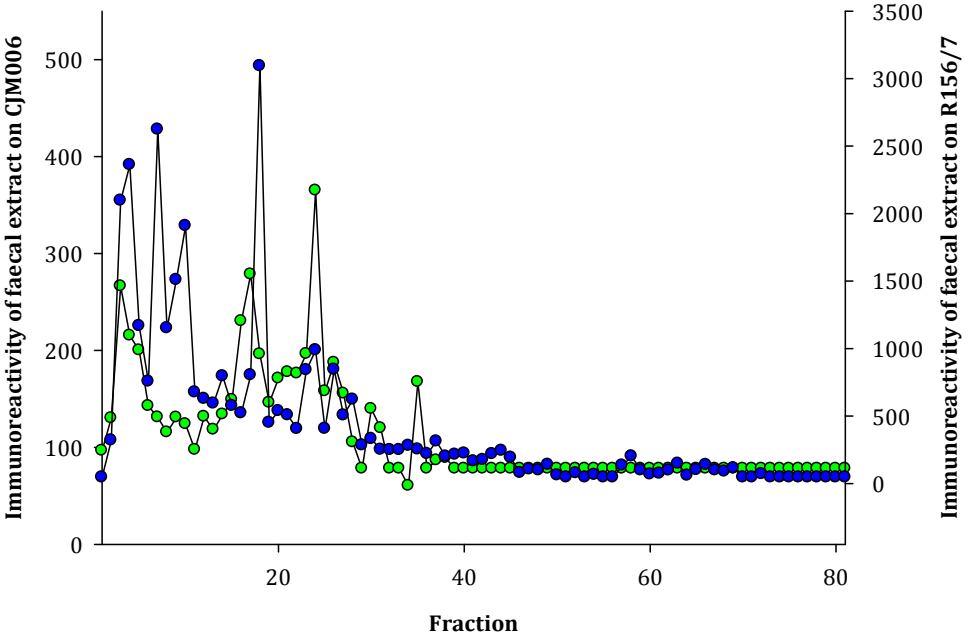
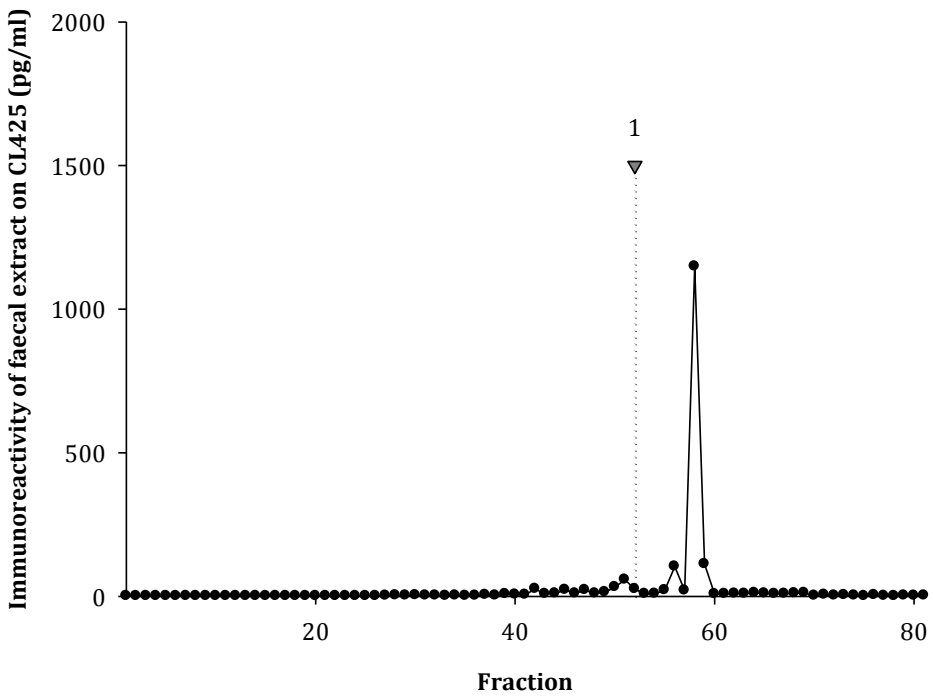


Figure 2.15: Immunoreactivity of male black rhino faecal extract, separated with (a) methanol and (b) acetonitrile gradient, on corticosterone EIA CJM006 (●) and testosterone EIA R156/7 (●).

2.4.3.4. Progesterone EIA CL425

Immunoreactivity profiles using both MeOH and MeCN separation show one clear peak at elution positions 58 and 38 respectively (Figure 2.16), with a number of smaller peaks at higher and lower retention times. Although there seems to be only a small amount of immunoreactivity at the same elution position as the synthetic progesterone standard (4-Pregnene-3, 20-dione) (52 and 35 respectively), the CL425 antibody is known to cross-react > 1% with 11 different P4 metabolites (Appendix 2), and it is likely that a slightly less polar metabolite of P4 is the main immunoreactivity peak seen in female black rhino faeces.

(a)



(b)

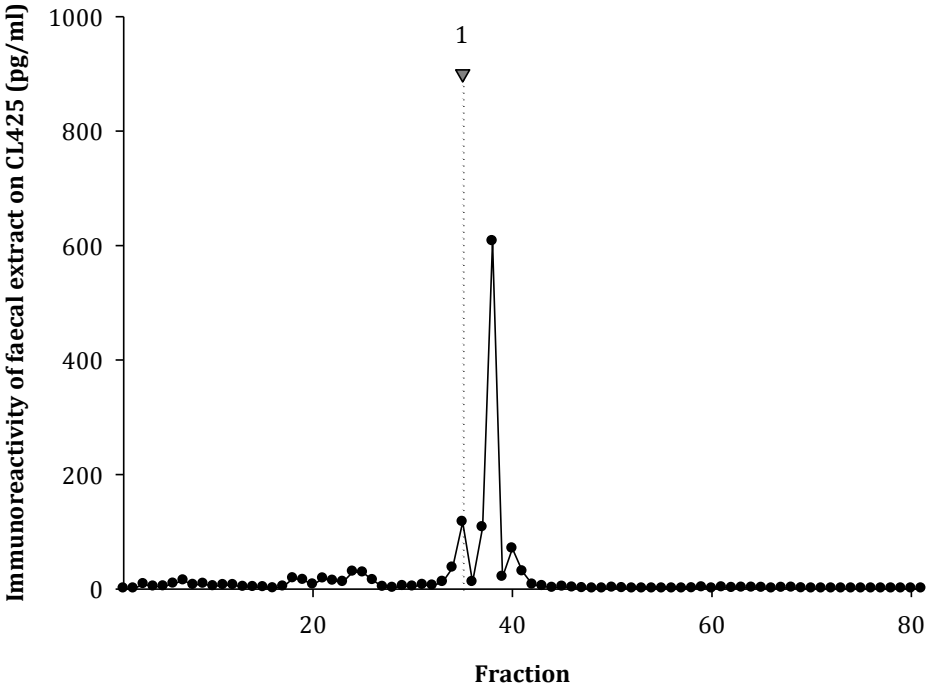


Figure 2.16: Immunoreactivity of female black rhino faecal extract, separated with (a) methanol and (b) acetonitrile gradient, on progesterone EIA CL425. Arrow (1) denotes elution position of synthetic progesterone (4-Pregnene-3, 20-dione).

2.4.3.5. Oestradiol EIA R4972

The oestradiol reference standards (oestradiol and oestrone) co-eluted at elution position 38 when using the methanol mobile phase, therefore only the acetonitrile separation was used for this assay. When these female black rhino extract fractions were run on the oestradiol EIA (R4972), there were three immunoreactivity peaks observed (Figure 2.17); one is likely to be a conjugate, as it was eluted almost immediately (elution position 3), indicating that it is more polar than the other compounds eluted. The other two immunoreactivity peaks observed in the faecal extract fractions eluted at 16 and 30 minutes. The first of these co-eluted with oestradiol-17β, whereas the other peak could not be identified based on the reference standards used, but is a less-polar compound than all of the reference standards used.

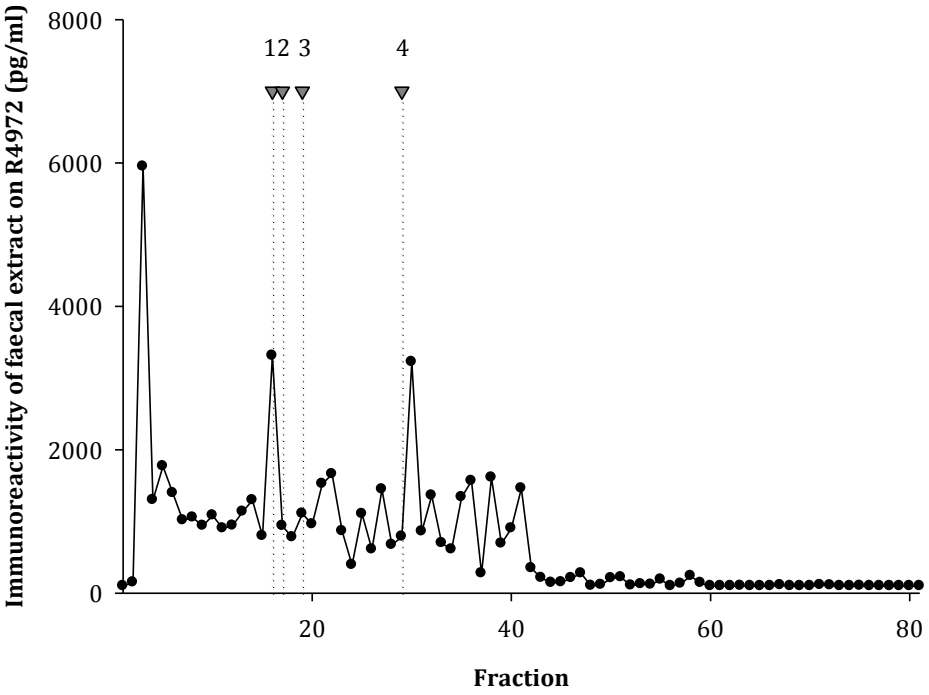


Figure 2.17: Immunoreactivity of female black rhino faecal extract, separated with acetonitrile gradient, on oestradiol EIA R4972. Arrows denote elution positions of synthetic standards 1) oestradiol-17β, 2) testosterone, 3) oestrone and 4) progesterone.

2.5. Biological validation

In addition to biochemically validating an EIA to ensure that the metabolites of interest are being accurately measured by the chosen assay, it is also important to ensure that those metabolites detectable in faeces are reflective of biological changes within the body. In order to do this, physiological validation is often used, such as an ACTH (Brown et al. 2001; Santymire et al. 2012) or GnRH challenge (Amaral et al. 2009; Chelini et al. 2011), which stimulate the hypothalamic-pituitary-adrenal axis, or the hypothalamic-pituitary-gonadal axis, resulting in the downstream production of glucocorticoids or reproductive hormones respectively, which can then be measured in faeces.

However, in the UK, scientific procedures including such physiological challenges are regulated under the Animals (Scientific Procedures) Act (1986). Conducting such procedures purely for research purposes would require a Home Office licence (Bishop et al. 2013), which was not deemed suitable for this study. Therefore for the purposes of this study, samples could only be utilised if such a procedure was already being carried out for veterinary purposes (RCVS 2012), or samples could be collected opportunistically elsewhere. As part of the validation for this study, we were fortunate enough to obtain faecal samples from a female black rhinoceros that required GnRH vaccination for a non-regulated procedure related to contraception, and archived faecal samples collected from four male black rhinoceros that had previously undergone a GnRH challenge at another institution (animal welfare and ethics for the procedure had previously been approved by the relevant local authorities; M. Bashaw, *pers. comm.*). These samples were therefore re-analysed in our laboratory, allowing us to assess the biological validity of hormone metabolites measured with our EIAs.

An alternative option is to use naturally occurring events that stimulate the desired response, which can be relatively easy to achieve in the case of adrenal activity, as stressors may occur either naturally, or as part of normal management practices. Biological validation has the added benefit that hormone metabolites measured in faeces are reflective of a natural response, so biologically relevant concentrations can be detected, as opposed to physiological challenges where the concentration of hormone metabolites measured in faeces is reflective of the amount of GnRH or ACTH injected.

2.5.1. Biological validation of corticosterone EIA for measuring adrenal activity in male and female black rhinoceros.

As it was not possible for us to perform an ACTH challenge as part of the validation process for this study, and no opportunities arose to utilise previously collected samples from elsewhere, it was necessary to use a biological validation. In this case, we have utilised inter-zoo transfers, during which 4 males and 5 females were moved from one zoological institution to another during the course of this study, as part of the European endangered species breeding program. Faecal samples were collected daily for 10 days prior to and 30 days following the transfer, which were frozen immediately following collection, and sent to Chester Zoo, UK for analysis as described in sections 2.2.3 and 2.2.4. Figure 2.18 illustrates a representative profile of faecal glucocorticoid metabolites prior to, and following an inter-zoo transfer in a female black rhinoceros.

In females, using a generalised linear mixed model (GLMM) controlling for multiple samples from multiple individuals (N=5), $\log_{10}$ transformed glucocorticoid metabolite concentration was significantly higher during the 30 days post-transfer than the 10 days pre-transfer (χ^2 =7.545, df=1, P=0.006; Figure 2.19), indicating that an increase in adrenal activity associated with this event could be detected in faecal samples.

However, in males (N=4), when controlling for repeated sampling within multiple individuals, there was no difference in $\log_{10}$ transformed glucocorticoid metabolite concentration before compared to after the translocation (pre 10 days vs. post 30 days, χ^2 =2.074, df= 1,P=0.15; Figure 2.19), and concentrations were in fact generally lower post-transfer. Although this does not follow the same trend as with the females, this does not necessarily mean that we are not measuring adrenal activity in male black rhino faeces. Indeed, Santymire et al. (2012) have previously demonstrated that this EIA (corticosterone CJM006) is capable of detecting the physiological response to an ACTH challenge in male black rhino. Instead, the lack of a response to the biological validation observed here may perhaps indicate a difference in how males and females respond to the potentially challenging event of an inter-zoo transfer, which requires further investigation. Furthermore, as all individuals were crate-trained prior to transfer (K. Edwards, *pers. obs.*), perhaps this event does not invoke the same response as a translocation *in situ* (Linklater et al. 2010).

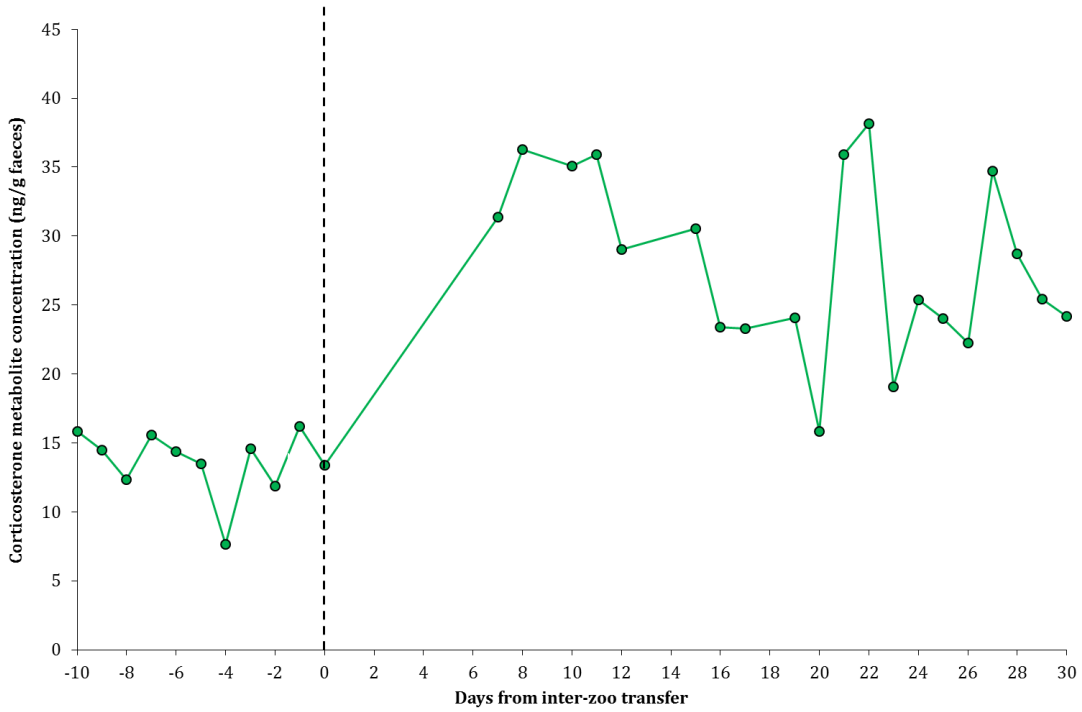
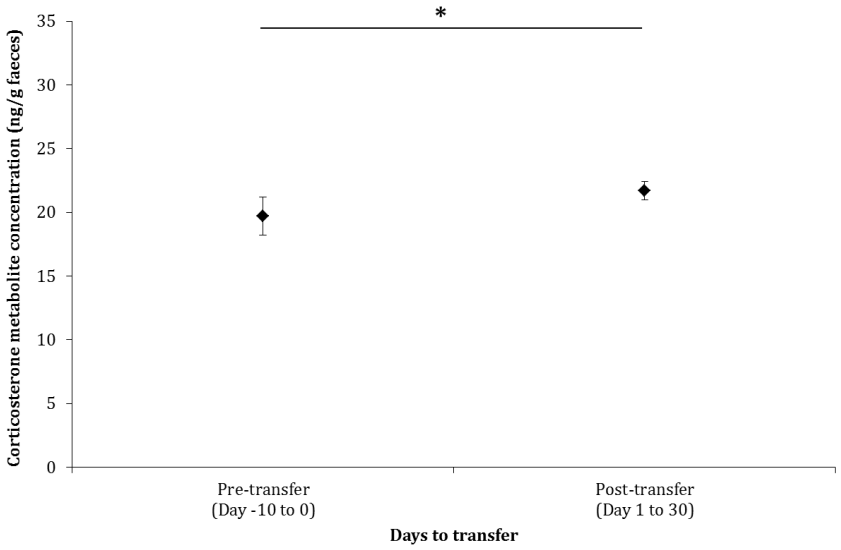


Figure 2.18: Faecal glucocorticoid metabolite concentration measured on the corticosterone CJM006 EIA following the inter-zoo transfer of a female black rhino. The dashed line represents the day of transfer, day 0.

(a)



(b)

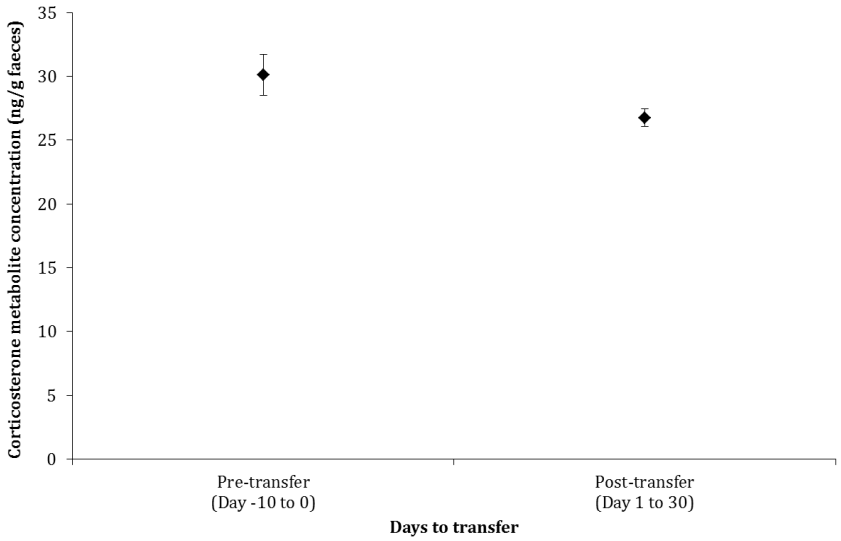


Figure 2.19: Average (± s.e.m) faecal glucocorticoid metabolite (fGCM) concentration in (a) five female and (b) four male black rhinoceros before compared to after inter-zoo translocation. When taking into account repeated sampling form multiple individuals in a GLMM, fGCM was significantly elevated in females during the 30 days post-transfer compared to the 10 days pre-transfer (*), but the reduction in fGCM observed in males was not significant.

2.5.2. Biological validation of the testosterone EIA for measuring male gonadal activity

As we were unable to conduct a GnRH challenge ourselves without a home-office licence, faecal samples that had already been collected by Taronga Western Plains Zoo, Dubbo, NSW, Australia, surrounding a series of GnRH challenges on four males previously conducted in 2005/6 were kindly provided for analysis. Archived faecal samples were shipped to Chester Zoo, UK for analysis following extraction and testosterone and corticosterone EIA protocols as previously described (sections 2.2.3 and 2.2.4). Figure 2.20 illustrates a representative profile of faecal testosterone and glucocorticoid metabolites prior to, and following a GnRH challenge in a male black rhinoceros.

Although the response to the GnRH challenge was variable among individuals, using a GLMM controlling for multiple samples from multiple individuals, testosterone metabolite concentration was significantly higher during the 5 days post-challenge than the 5 days pre-challenge (χ^2 =8.379, df=1, P=0.004), and showed a tendency (χ^2 =3.735, df=1, P=0.053) of returning to pre-challenge concentrations during the subsequent 5 days. However, no increase in corticosterone metabolite concentration was observed over the same period (pre 5 days vs. post 5 days, χ^2 =2.056, df=1, P=0.15; post 5 days vs. post 10 days, χ^2 =0.021, df=1, P=0.88) (Figure 2.21).

This indicates that a biological response to the GnRH challenge was detected using the testosterone EIA, with an increase in testosterone metabolite concentration as expected. However, a similar response was not observed in faecal glucocorticoid concentration, indicating that any potential cross-reactivity between these two EIA is minimal, and should not confound potential results obtained using these two assays. Furthermore, faecal samples collected as part of this study have demonstrated that faecal testosterone metabolite concentration increases with age, being significantly lower in immature than mature males (Chapter 5). This also acts as a form of biological validation, as testosterone production would be expected to increase with sexual maturity (August et al. 1972), and this was reflected in the faecal metabolites measured by this EIA.

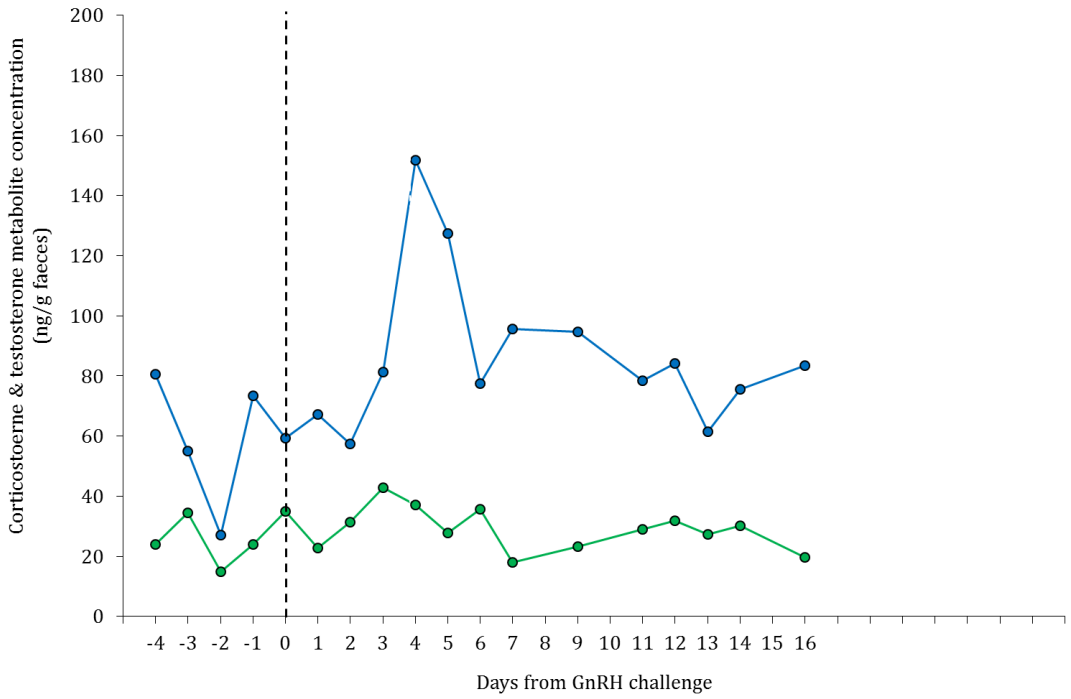


Figure 2.20: Faecal testosterone (●) and corticosterone (●) metabolite concentration for 4 days prior to and 16 days following a GnRH challenge in a male black rhinoceros. The dashed line represents the day of GnRH challenge, day 0.

(a)

(b)

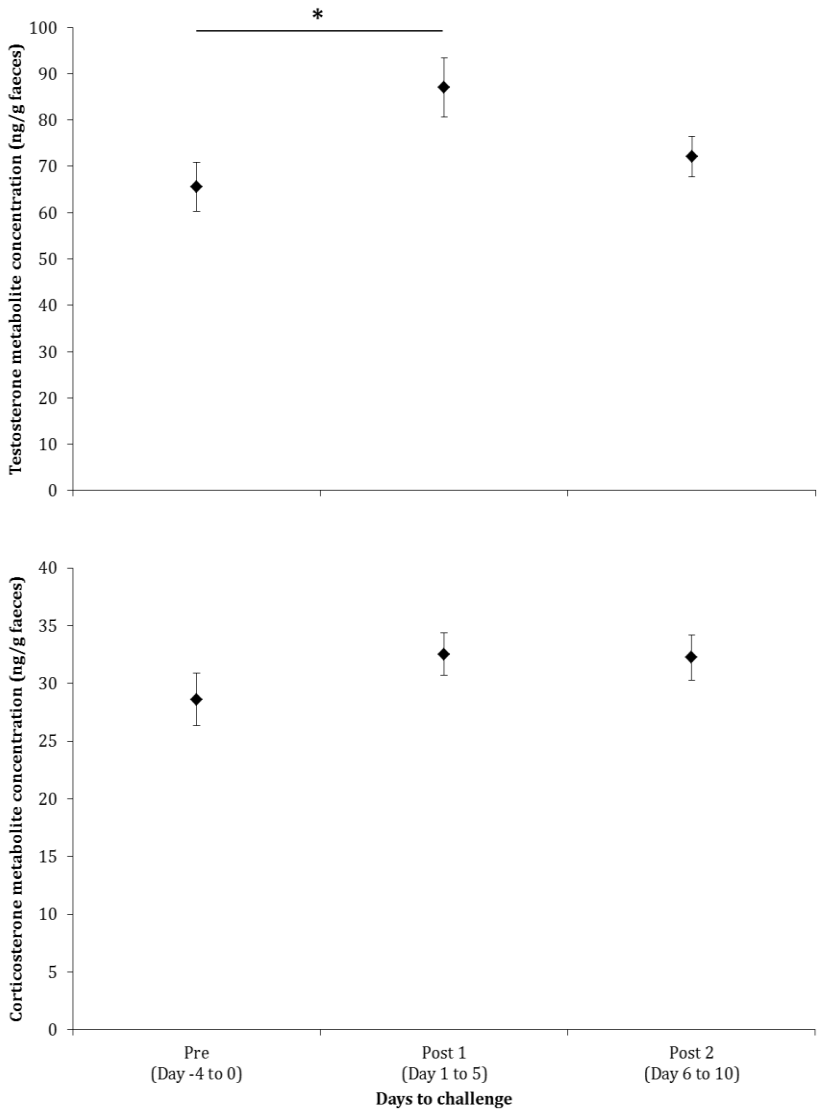


Figure 2.21: Average faecal testosterone (a) and corticosterone (b) metabolite concentration (± s.e.m) in four black rhinos before and after a GnRH challenge. Annotation (*) represents a statistically significant difference ($P<0.05$) between pre-challenge samples (days -4 to 0) and post-challenge samples (days 1 to 5) in testosterone, but not in corticosterone.

2.5.3. Biological validation of progesterone and oestradiol EIAs for measuring female gonadal activity.

A 13 year old female black rhinoceros at Chester Zoo, UK developed a number of reproductive pathologies, and for her on-going health and welfare, it was decided that a contraceptive treatment using a GnRH vaccination regime was required in an attempt to minimise further development of her condition (R. Hermes, *pers. comm.*). As the GnRH vaccine used contains a GnRH protein conjugate, it initially acts in a similar way to a GnRH challenge, stimulating the HPG axis. Subsequently the immune system develops anti-GnRH antibodies to neutralise endogenous GnRH, blocking its biological activity, reducing the secretion of LH, FSH and sex hormones, to shut down the reproductive system (Feltrer 2010; Kirkpatrick et al. 2011). Therefore, the veterinary requirement to carry out this treatment was opportunistically used as a physiological validation to assess the hormone metabolites measured in faeces. Faecal samples were collected from this female prior to, and following the treatments, and were extracted and analysed on progesterone and oestradiol EIAs according to previously described protocols (sections 2.2.4.1-2.2.4.2).

Figure 2.22 illustrates faecal progesterone and oestradiol metabolites prior to, and following treatment with a GnRH vaccine in this female. When samples were divided into five day periods (pre: days -4 to 0; post 1: days1-5; post 2: days 6-10; and post 3: days 11-15), progesterone metabolites were significantly elevated during the 5 days following treatment (post 1 vs. pre: χ^2 =5.807, df=1, P=0.016). During the following 10 days, faecal progesterone metabolites were then suppressed below pre-treatment concentration, and were significantly lower than the initial 5 days following treatment (post 2 vs. post 1: χ^2 =13.499, df=1,P<0.001; post 3 vs. post 1: χ^2 =11.174, df=1,P<0.001; Figure 2.23). Faecal oestradiol concentration was also increased during the first 5 days following treatment, although this did not quite reach significance (post 1 vs. pre: χ^2 =3.436, df=1, P=0.064). During the following 5 days, faecal oestradiol concentration was suppressed compared to the initial 5 days following treatment (post 2 vs. post 1: χ^2 =4.217, df=1, P=0.04; Figure 2.23), returning to around pre-treatment concentration. This indicates that a biological response to the GnRH treatment was detected using the progesterone, and to a lesser degree the oestradiol EIAs, with an increase in metabolite concentration observed as expected.

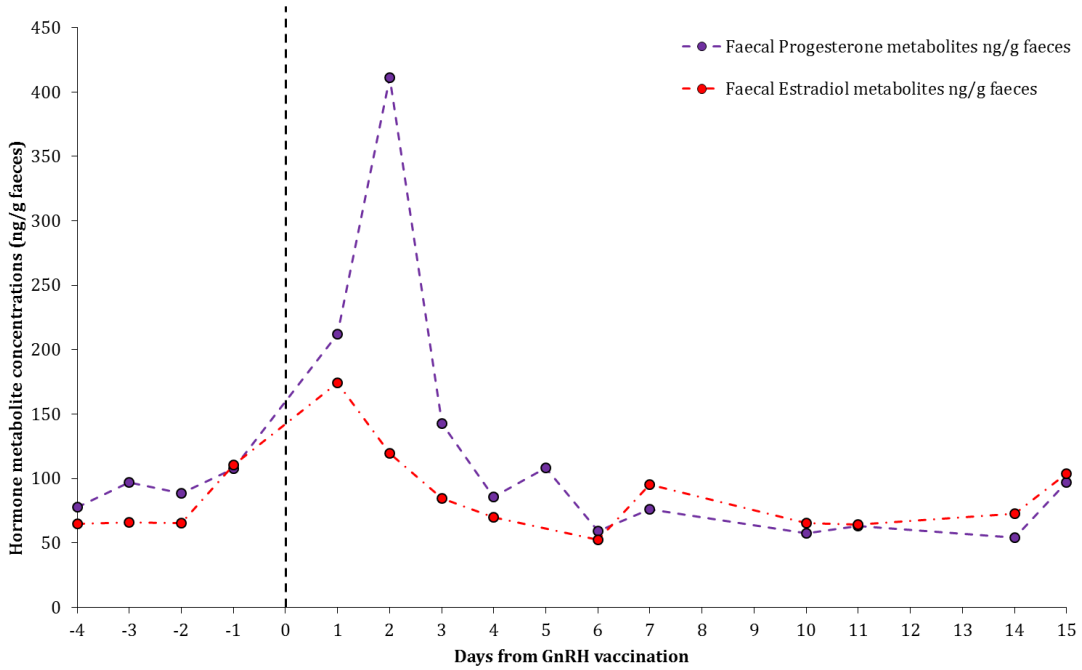
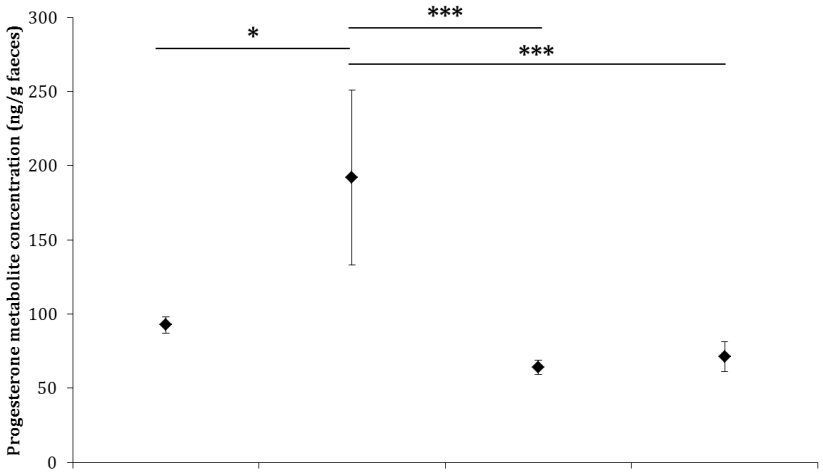


Figure 2.22: Faecal hormone metabolite concentrations measured on progesterone (●) and oestradiol (●) EIAs following GnRH vaccination for contraception purposes in a single female black rhino.

Furthermore, by way of biological validation of the progesterone and oestradiol EIAs, samples collected as part of this study have indicated two things. Firstly, cyclic changes in progesterone metabolite concentration have been detected using the progesterone CL425 EIA (Chapter 5). This cycle pattern is reflective of progesterone production from the corpus luteum following ovulation, which then regresses and ceases progesterone production in preparation for the next period of oestrus. Secondly, oestradiol metabolite concentration as determined using the oestradiol R4972 EIA was significantly lower during periods of acyclicity than during normal oestrous cycles (Chapter 7). This indicates that the absence of oestradiol metabolites detected in faeces may be reflective of reduced follicular activity during acyclic periods.

(a)



(b)

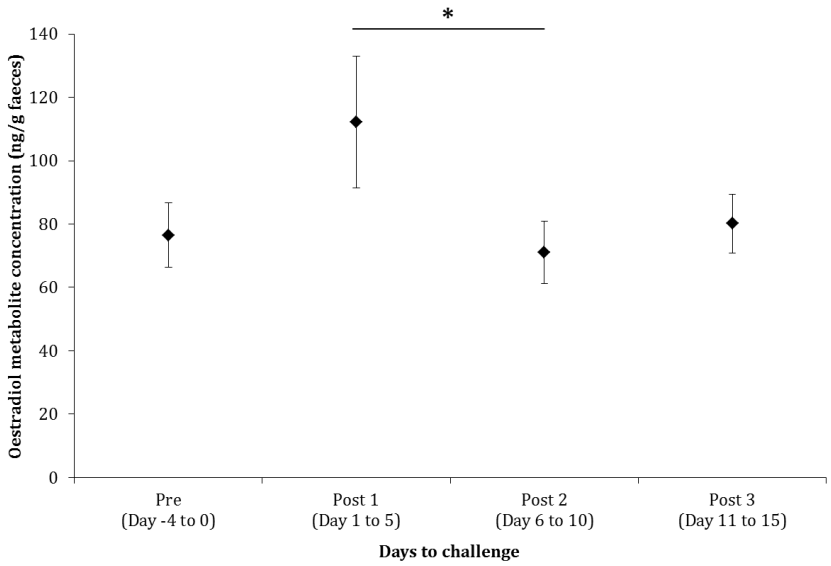


Figure 2.23: Average hormone metabolite concentrations (± s.e.m) in faecal extracts from a single female black rhino before and after a GnRH vaccination. Extracts were run on a) progesterone and b) oestradiol EIAs. Annotations represent statistically significant differences (* P<0.05; *** P<0.001) between pre-challenge samples (days -4 to 0) and post-challenge samples (days 1 to 5; 6 to 10 and 11 to 15).

2.6. Conclusion

- Endocrinology can be a useful tool to help understand the physiology behind processes such as reproduction, response to potential stressors, and behaviour.
- In order to conduct the proposed research on the black rhinoceros, where longitudinal sampling was important, a practical, non-invasive method of sample collection was required.
- Enzyme immunoassays to measure progesterone, oestradiol, testosterone and glucocorticoid metabolites have been biochemically validated to measure hormone metabolites in the faeces of male and female black rhinoceros through illustration of parallel displacement curves and no evidence of matrix interference.
- To ensure reliability of data for use in this study, modifications were made to the corticosterone and testosterone EIA protocols used, to ensure variation due to environmental effects was kept to a minimum.
- High-performance liquid chromatography (HPLC) separation of faecal extracts revealed a number of immunoreactive peaks on each EIA, some of which co-eluted with reference standards, indicating that although a number of metabolites are being detected, they are immunologically similar to the hormone to which the EIA was developed.
- Where possible, biological validation was also conducted, to confirm that hormone metabolites measured in faeces were indeed reflective of biological changes.

2.7. Acknowledgements

- Sue Walker for setting up the EIA's and for her tuition and guidance
- Coralie Munro for providing reagents, advice and support
- International Society of Wildlife Endocrinology (ISWE) members for helpful advice, particularly on troubleshooting
- The numerous technicians and interns at Chester Zoo for help with extracting faecal samples:
    - Thijs van den Houten
    - Rebecca Watson – particularly for corticosterone development, whose M.Sci dissertation was based on troubleshooting this EIA
    - Rebecca Purcell
    - Vicki Norton– particularly for testosterone development
    - Hannah McArthur
    - Jenny Hardy– particularly for a long summer extracting faecal samples
    - Aaron Marshall
    - Leanne Harrington
    - Taylor Harrison
    - Katherine Cho
    - Saran Crosby
    - Katja Kaikko
    - Lois Byrom
- Taronga Western Plains Zoo, Dubbo, New South Wales, Australia (Meredith Bashaw, Rebecca Spindler and Tamara Keeley) for providing their archived faecal samples allowing us to conduct the biological validation without being able to conduct a GnRH challenge.
- Thermo Scientific for all their help and support with HPLC, especially Monica Dolci, Louisa Pereira, Tony Edge and the technical support team, Tim Liddicoat and Harald Ritchie.

CHAPTER 3

3. POPULATION PERFORMANCE OF *IN SITU* EASTERN BLACK RHINOCEROS (*DICEROS BICORNIS MICHAELI*) IN KENYA.

Summary

Following an estimated 96% decline in black rhino numbers across Africa, biological management of remaining populations has become essential, not only to halt further decline, but also to maximise population growth and genetic diversity. Demographic monitoring of these populations allows information to be gained on performance, both at the individual and population level, which can be used to guide management decisions to maximise metapopulation growth and viability. Demographic information from eight populations of eastern black rhinoceros in Kenya were used to fulfil the two main aims of this chapter. Firstly, historical data was used to create simulated population projection models, to determine the current performance of *in situ* populations, and how growth rates vary under natural conditions. Secondly, data were used to estimate indicators of population performance relating to mortality, reproduction and population structure, to establish the normal range and variability both between individuals and across reserves. Projected growth rates across reserves ranged from 2.26% to 7.02%, with only five of the eight reserves currently predicted to achieve the target of 5% growth per annum. Although mortality and population structure indicators were generally achieved, indicators relating to individual reproductive success were often sub-optimal. This information provides a better understanding of how this species is performing *in situ*, which is not only beneficial to the biological management these populations, but can also be used as a biological reference for the *ex situ* population to determine where there may be room for improvement (see Chapter 4). If black rhinoceros populations are to be managed effectively, both *in situ* and *ex situ*, it is essential to gain a better understanding of measures of individual and population performance, and how demographic parameters can be used as indicators for where improvements could be made.

3.1. Introduction

The eastern black rhinoceros (*Diceros bicornis michaeli*) was once numerous in Kenya, with an estimated population of 20,000 individuals in 1970; the larger reserves such as Tsavo National Park and Selous Game Reserve each containing perhaps twice the current global population of all three remaining subspecies of black rhino combined (KWS 2012). However, by 1990, less than 400 individuals remained in Kenya, predominantly due to the wide scale poaching occurring across the continent (Bradley Martin and Bradley Martin 1982). Populations were left vulnerable and highly fragmented, so individuals were captured and translocated into designated reserves, where security could be concentrated to protect populations against further decline (Emslie and Brooks 1999). As a result, numbers have steadily increased over the last two decades (Figure 3.1); Kenya remains the stronghold for the eastern subspecies, with 623 individuals at the end of 2011, 80.3% of the remaining wild population (KWS 2012). Other significant populations of the eastern subspecies include two populations in Tanzania, and an out of range population in South Africa.

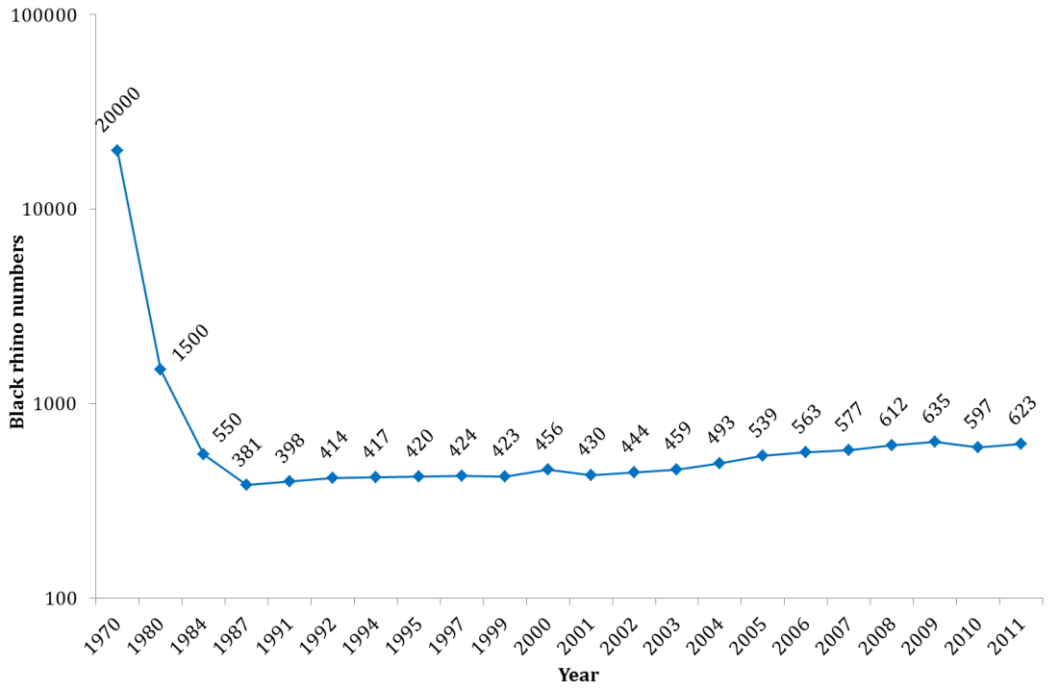


Figure 3.1: Eastern black rhino numbers in Kenya from 1970 to 2011, shown on a logarithmic scale. After a rapid decline between 1970 and the mid 1980's, Kenya's black rhino populations have been slowly increasing, from a minimum size of 381 in 1987 up to 623 by the end of 2011. Adapted from (KWS 2012) with permission from KWS rhino programme.

However, poaching remains a threat to the survival of rhinoceros species globally, with a significant resurgence over recent years (Emslie et al. 2012), threatening the positive trends in population growth achieved over the last two decades. This has been particularly severe in southern Africa, but has also led to the loss of 53 eastern black rhinos in Kenya between 2007 and 2011, when 2.3% of the existing rhino population was lost to poaching within a 12-month period (KWS 2012). With this continuing threat, the future of the black rhino is heavily dependent on the biological management of remaining populations, to maximise the rate of growth and ensure genetically and demographically viable populations, and continuing to expand their current range, whilst maintaining suitable levels of protection (KWS 2012).

The key to the success of black rhino conservation over the last two decades has been the intensive biological management and protection of remaining populations, and the translocation of surplus individuals to establish new populations elsewhere in their native range. The aim of biological management is to maintain rapid population growth, and to minimise inbreeding and maximise genetic diversity through the translocation of rhinos between reserves. Due to security demands, rhino populations are maintained within small and mostly fenced reserves (Okita-Ouma et al. 2010), and as such, each reserve has a finite ecological carrying capacity that can be supported, before detrimental effects on growth may occur (Adcock 2001). Black rhinos within these reserves are ideally managed at the 'maximum productivity carrying capacity' (Adcock 2001; KWS 2012), generally around 75% of the ecological carrying capacity, meaning translocations may be necessary to relieve pressure on existing populations (Patton et al. 2008), to prevent any decline in growth.

The IUCN African rhino specialist group (AfRSG) has proposed a minimum metapopulation growth rate of 5%, with well-established populations in good habitat hoped to attain 6.5-9% growth per annum, or even higher for young breeding populations with low mortality (KWS 2012). This growth rate has been specified to promote rapid growth and minimise the loss of genetic diversity from the remaining populations. Kenya's long-term vision is to maintain 2000 black rhinos nationally, and in the shorter term, to achieve 750 black rhinos in Kenya alone by 2016. To achieve these goals, at least 5% annual growth rates must be maintained both nationally, and within each individual reserve, and man-induced and disease-related deaths must be limited to less than 1% of the population (KWS 2012). High biological growth rates result from good breeding performance; however, this is heavily dependent on

conditions such as habitat quality (Okita-Ouma et al. 2008), and population density (Patton et al. 2008), and on demographic parameters such as sex ratio (Okita-Ouma et al. 2010) and age structure of the population (Hrabar and du Toit 2005). In order to maintain such high rates of growth, demographic monitoring is essential to understand population performance and prevent any decline in population growth (Emslie 2001b), either within individual reserves, or at a national level.

A number of performance indicators have been established (du Toit et al. 2001) to assess individual and population performance in parameters such as age at first birth, inter-calving intervals, what proportion of the population breed each year, age and sex structure of the population, and mortality. However, it is not only important to assess performance against these optimal targets, but also to investigate why certain individuals or sub-populations may fail to meet them. On-going population monitoring of past and current trends and the projection of likely future performance using population viability analysis (PVA) based on structured demographic data can be beneficial to management, as it can give an indication of overall performance, but also allow identification of areas that may not be performing as expected. This also allows an understanding of the variability in demographic parameters, and how these may be related to environmental constraints or social structure, which can be beneficial when establishing new populations.

This variability in performance is important to understand differences within reserves over time and between reserves, but also to understand differences between individuals in their contribution to population growth. If a population is to achieve the necessary targets for growth, and to retain maximum genetic diversity for the future, it is important that all individuals are reproducing equally (Muya et al. 2011). Using individual measures of reproduction, such as age at first birth and inter-birth intervals, it is possible to determine whether breeding females are performing optimally, but it is also important to determine the number of males and females that are not reproducing. Skewed reproductive output across individuals has implications for overall growth rates, as although a proportion of the population may be achieving the necessary targets for 5% growth, any non-reproducing individuals will reduce the percentage growth that can be achieved. Furthermore, reproductive skew also has implications for the genetic diversity of the population, as the difference between effective population size (N_e) of breeding individuals, and overall population size has implications for the rate of loss of genetic diversity within finite populations (Frankham 1995).

Conservation of the black rhinoceros requires a global strategy, with *in situ* and *ex situ* populations both playing a role in long-term viability of this species (Emslie and Brooks 1999; Foose 1993). As such, a better understanding of black rhino demography can also be beneficial to *ex situ* conservation, where inconsistent rates of reproduction and high rates of mortality have been limiting population growth (Emslie and Brooks 1999; Smith and Read 1992). There are two aims of this chapter; firstly, to determine the current performance of *in situ* eastern black rhinoceros and how population performance varies across reserves; and secondly, to generate demographic information compiled on these populations, to determine how performance indicators may vary between individuals. It is important to gain a better understanding of how this species is performing *in situ*, to establish baseline performance indicators, and the variability in these measures both between individuals and across reserves. This information can not only be beneficial to the biological management of *in situ* populations, but can also be used as a biological reference for the *ex situ* population, to determine where there may be room for improvement (see Chapter 4). If black rhinoceros populations are to be managed effectively, both *in situ* and *ex situ*, it is essential to gain a better understanding of individual and population performance, and how demographic parameters can be used as indicators for where improvements could be made.

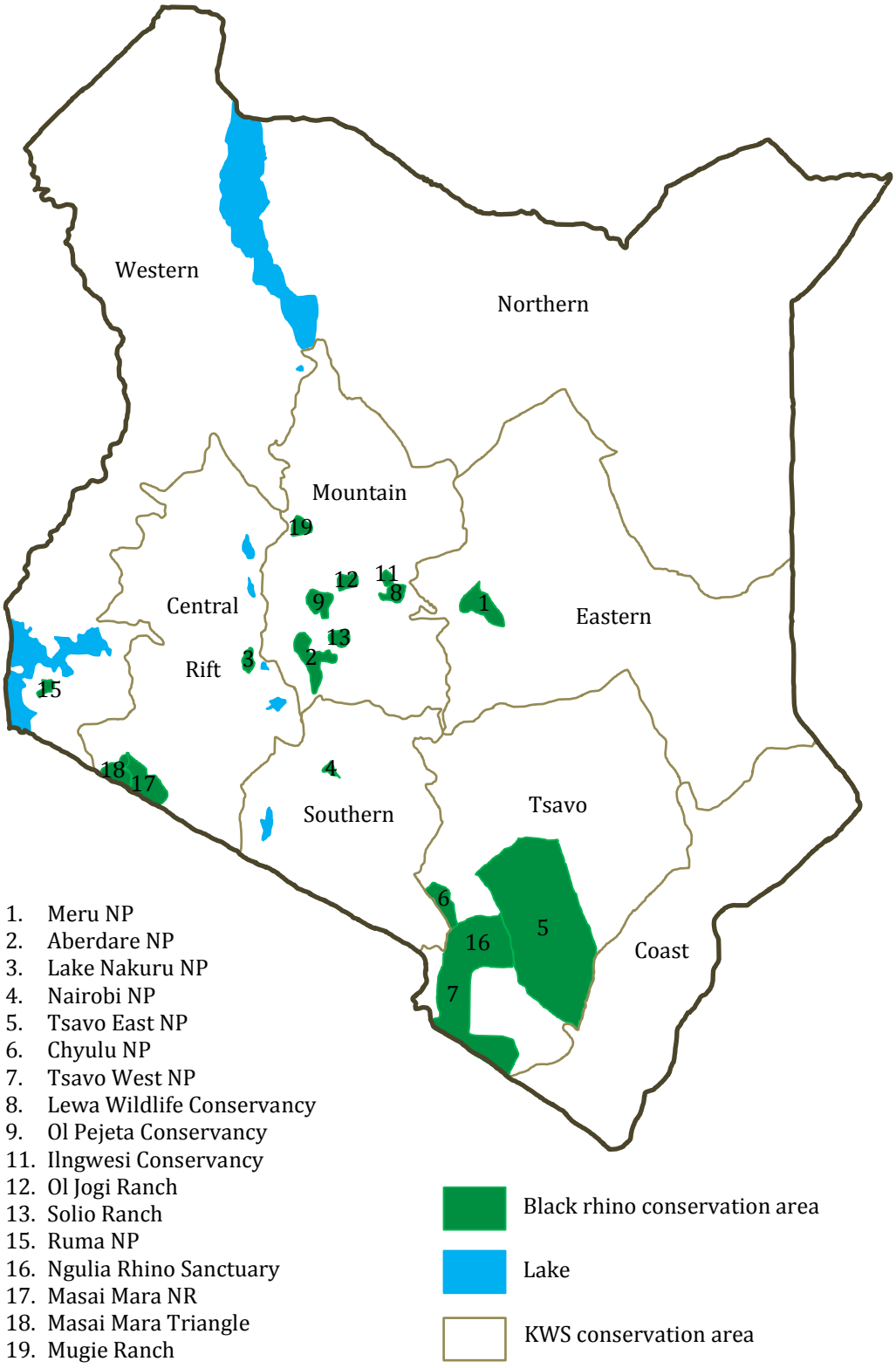


Figure 3.2: Distribution of black rhino conservation areas in Kenya, 2011. Adapted from (KWS 2012) with permission from KWS rhino programme.

3.2. Methods

3.2.1. Study populations and demographic information

Data were compiled on rhinos at eight reserves across Kenya (Figure 3.2), managed by Kenya Wildlife Service (KWS), as described in Table 3.1. Data were only used for analysis if monitoring records were sufficiently complete for a given year; resulting in data being truncated for Masai Mara (1984-2008) and Nairobi (1985-2010), and for Ol Jogi (1989-2010; prior to this there were only three founders on the reserve, so parameters were excluded from this period due to small sample size). For all other reserves, records began at reserve establishment, and were current up to 2010 or 2011. Additionally, for the seven reserves that were established prior to 2000, a shorter time-frame of the latest 10-years was used in addition, to give an indication of more recent population trends (see Table 3.3 for time-frames used).

For relatively small populations of long-lived species, year-to-year differences in age-specific vital rates due to demographic stochasticity (random fluctuations in reproduction and survival due to chance, which are emphasised in small populations) may be quite apparent. The benefit of using longer time frames of demographic parameters is that these stochastic effects are smoothed out over time, giving a more reliable measure. However, these longer time-frames do not necessarily reflect changes in growth over time, for instance whether growth rates have improved or declined in recent years; therefore a more current perspective is gained by using shorter time-frames. Since both approaches have potential advantages and disadvantages, using the two in parallel, allow a more robust prediction of population growth. Further details about each reserve can be found in (Brett 1993; KWS 2012; Okita-Ouma et al. 2007).

Information was compiled by KWS staff, using the Kenya Black Rhino Information Management System (KIFARU©); including all translocations, births and deaths of rhinos in the eight reserves. This information was transferred into Microsoft Excel®, condensed into individual records and checked for errors, missing data, or duplication. Any queries were clarified with KWS staff, and using previous records or published data. For each individual within the dataset, a date of birth (approximate or known) was determined, to allow calculation of either current age or age at death. Where known, the mother (dam) and father (sire) of each calf born was recorded, to allow calculation of the age at calving (age of dam when she gave birth to calf) or conception (age of sire when female conceived), and for use in calculation of female age-specific fecundity (See section 3.2.2). For any cases where the dam was unknown, the birth was

included for calculations of percentage adult females breeding per year, but not included in age-specific fecundity calculations. Demographic data were compiled separately for each reserve, and any individuals translocated between reserves were recorded in the relevant reserve according to the date of translocation. Data was then summarised for each reserve, and detailed population history tables produced in order to calculate vital rates for population modelling (See section 3.2.2).

Table 3.1: Name, year of establishment and approximate area of designated rhino sanctuaries, where either the entire park or a sub-section has been set-aside for this purpose. Further description of the location and habitat of each of the parks can be found in the provided references.

Name of reserve (rhino sanctuary within)	[a] Year established	[b] Area (km^2)	Ring-fenced	Additional information	References
Lewa Downs Conservancy	1984	247	Yes	Ngare Sergoi Rhino Sanctuary extended to incorporate Lewa Downs (1988) and Ngare Ndare Forest Reserve (1991).	(Merz 1991)
Masai Mara Nature Reserve (Masai Mara triangle)	1948	1510	No	Masai Mara complex (Masai Mara Nature Reserve and Triangle) adjoins the Serengeti - free movement of rhinos between areas.	(Morgan-Davies 1996; Walpole et al. 2001)
Mugie Ranch (Mugie Rhino Sanctuary)	2004	89	Yes	Ceased to be a rhino sanctuary in 2012 following relocation of rhinos due to elevated risk of poaching in the area.	(KWS 2012; Okita-Ouma et al. 2007)
Nairobi National Park	1963	117	Partially	National Park since 1945; rhino sanctuary since 1963	(Muya and Oguge 2000)
Lake Nakuru National Park	1987	144	Yes	Total size 188km^2, of which 44km^2 is the lake	(Mwangi and Western 1998)
Tsavo West National Park (Ngulia Rhino Sanctuary)	1986	92	Yes	Situated within Tsavo West NP but separated by fence line.	(Okita-Ouma et al. 2008)
Ol Jogi Conservancy (Ol Jogi Ranch & Pyramid Black Rhino Sanctuary)	1979	249 (Pyramid 50)	Yes	Pyramid reserve and main ranch separated by fence line, rhinos in both sections but no free movement between areas.	(Ndeereh et al. 2012)
Ol Pejeta Conservancy	1989	300	Yes	Ol Pejeta extended in 2007.	(Patton et al. 2010a, b)

[a] Year established as designated rhino sanctuary, park may have existed prior to this; [b] The area refers to that which is used as a designated rhino sanctuary, and may be located within a larger Park area; sizes taken from Okita-Ouma (2004) and Muya et al. (2011).

3.2.2. Population modelling

Population viability analysis was carried out for each reserve, using a single-sex, female-based transition matrix model constructed in MATLAB® (The MathWorks Inc 2008) using code adapted from Morris and Doak (2002) (See Appendix 4 for examples of code used). This method allowed model parameters to be specified to suit this species, and the type of data available. In this case, the published code was adapted to incorporate six age classes, which were based on similarities in vital rates. Using these vital rates, the matrix elements were specified (KEmxdef.m, Appendix 4), and the distributions used to simulate the different vital rates were specified to suit the life history of this species, as detailed below. Data were calculated 1) using the entire time-frame available (all eight reserves), and 2) using the last ten-year time period (except for Mugie established in 2004). The transition matrix, *A*, contained six age-classes, and represents the potential contribution by each class *j* individual to the population at the next census.

Age-specific fecundity was calculated as the number of same sex offspring born to females aged *x,* divided by the total number of females at risk of giving birth at age *x* in the population during a given year. Age-specific mortality was calculated as the number of females that died age *x,* divided by the total number of females at risk of dying at age *x* in the population during a given year. Mortality was converted to age-specific survival, as 1-mortality. Due to the small population size, and long-lived nature of this species, females were divided into six age-classes based on similarities in age-specific fecundity and mortality. These six classes included individuals aged 0-1, 1-5, 5-9, 9-17, 17-33 and 33+, and the transition probability of individuals in one class at time *t* progressing to the next class by time *t+1* was also calculated. Models were performed based on no further imports or exports, to give a prediction of the natural growth rate of each population due to births and deaths alone.

The vital rates of fecundity (f1-6), survival (s1-6) and transition probability (g1-6) were calculated for each age-class, for each year, and the mean and variance in each rate calculated between years. In two reserves, the calculated variance in survival vital rates were considered to be unrealistic due to the relatively small number of females that had been at risk in these age classes during the study period (Mugie, s_1 and Ol Pejeta s_6). In these cases, the variance was adjusted to a more conservative estimate based on the variance values calculated for those particular vital rates from the other reserves. The vital rates were then transformed into matrix elements to reflect the

potential contributions an average individual in each class would contribute to all other classes in the following census.

In the black rhinoceros, where reproduction can occur at any point throughout the year, and a maximum of one offspring is produced during an inter-census interval (Estes 1991), fecundity can be interpreted as a probability. The contribution through reproduction by each class *j* individual to the population at the next census therefore consists of i) the probability that a class *j* individual survives to the midpoint of the inter-census interval ($\sqrt{s_j}$), ii) the probability that a class *j* individual will reproduce (f_j), and iii) the probability that the produced offspring will survive to the following census ($\sqrt{s_1}$). Individuals in class *j c*an therefore contribute to the population at the following census (*t + 1*) by 1) surviving and progressing to the next age class, $s_j\, g_j$, or 2) surviving and staying in the same age class, s_j (1-g_j), and 3) through reproduction $f_j\ \sqrt{s_j}\ \sqrt{s_1}$ (Figure 3.3).

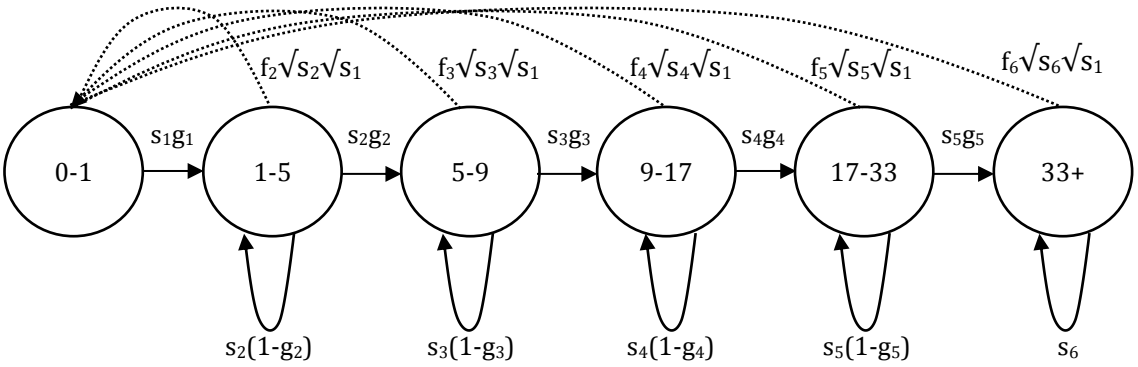


Figure 3.3: Schematic diagram of the potential contribution by female black rhinoceros in six age-classes at time *t* to the population at time *t+1*. Dashed lines represent the recruitment of new individuals into the population through reproduction; solid lines represent the individuals moving between, or remaining within age classes. s_j is the survival rate for individuals in class *j,* g_j is the probability that an individual in class *j* transfers to class *j+1* by the following census, and f_j is the rate of fecundity of individuals in class *j*.

Stochastic simulations were conducted using mean vital rates and variance calculated from raw demographic data from each population. To incorporate this variance into the model, for each year of the projection, the vital rates were simulated from a set of random numbers taken from the beta distribution, specified by the observed mean and variance for each vital rate. The beta distribution was selected for the simulation in this

species, as both survival and fecundity represent probabilities. The vital rates were converted into matrix elements as described above, and the resulting transition matrix was multiplied by the population vector, *n*, to give the population size the following year. This was repeated as single-year time steps for 100 years, and each simulation was repeated 1000 times, to allow good representation of parameter combinations and produce a reliable estimate of future population size and growth rate. If the quasi-extinction threshold of 20 individuals (or 0 for smaller populations) was met within a given simulation, the model moved onto the next iteration, and the probability of extinction was calculated for the population. The simulation also included a deterministic projection, using the average vital rates to calculate λ_1, the dominant eigenvalue of transition matrix *A*.

3.2.3. Demographic performance indicators

Effective biological management of black rhinoceros populations requires on-going monitoring to determine whether they are achieving the specified target of >5% growth per annum. A number of performance indicators have been established, which can be used to assess whether individual populations are performing as required. These indicators were discussed during a SADC (Southern African Development Community) rhino management group (RMG) workshop on biological management to meet continental and national black rhino conservation goals, in 2001 (du Toit et al. 2001; du Toit 2001; Knight and Emslie 2001) and benchmarks established for each of these parameters (see Table 3.2). The following performance indicators were calculated for each reserve separately, and compared between reserves with respect to growth rate, and to the pre-determined targets.

- ***Age at first reproduction* (*birth*) (*females*)*: Below 7 years is optimal, although can be as young as 4-5.*** Age at first birth includes only calves viable at birth, does not include premature or stillborn calves, or mid-term abortion. This indicator provides a good individual measure, but requires detailed monitoring and accurate dates of birth, and can be misleading if neonatal deaths are not observed.

- ***Age at first reproduction* (*conception*) (*males*)*: no benchmark established.*** Age at first conception represents the age of the male when a female conceived

– i.e. the first known successful mating resulting in the birth of a viable calf, and is estimated as 450 days prior to birth. Males are generally considered to be sexually mature at 7-10 years, around the age at which they may become territorial (Garnier et al. 2001). However, anecdotal reports suggest that males have been known to sire earlier than this (*ex situ* 4-5 years (EEP studbook, *pers. obs.*); *in situ* 4 years 5 months (Goddard 1970) 6yrs (Hall-Martin 1986),). Spermatogenesis commences at 7-8 years (Hitchins and Anderson 1983).

- ***Inter-birth interval* (*IBI*) (*females*)*: With a gestation of 15-16 months, an inter-calving interval of less than 3 years is good.*** Hormone data suggest that post-partum anoestrous may occur, lasting between 3-10 months (Brown et al. 2001; Garnier et al. 2002) and *pers. obs.*), but with lactation lasting on average 18-24 months, females are able to conceive prior to weaning. This indicator also provides a good individual measure and correlates well with the percentage of females breeding per annum. However, this requires detailed monitoring of individual females over long time-frames, and can become misleading if post-natal deaths are not detected.

- ***Percentage of adults breeding per annum* (*females*)*: Greater than 30% is good.*** This indicator correlates well with inter-calving interval, assuming all adult females in the population are breeding. However, it also requires detailed monitoring of females, and can become misleading if post-natal deaths are not detected. Especially in small populations, this measure should be averaged over 3-year windows to allow for synchronised calving.

- ***Percentage of adults breeding per annum* (*males*)*: no benchmark established.*** Black rhinos have a polygynous mating system, with dominant males defending access to receptive females in their territory from subordinate males, therefore some degree of reproductive skew in males is to be expected (Garnier et al. 2001). However, minimising skew through management of males is beneficial for genetic diversity of the population (Muya et al. 2011).

- ***Mortality* (*males and females*)*: Whole population <4%, calves* (*0-1yr*) *<10%, sub-adults <5%.*** This indicator provides a true reflection of a problem, although once deaths have occurred, the cause of the problem may already be difficult to remove through management. This indicator requires averaging

over multiple years to allow for catastrophes e.g. drought or disease, and thorough monitoring to distinguish 'not-seen' and 'known-to-have-died', especially in reserves with incomplete fencing, and to distinguish between natural and unnatural mortality.

- ***Adult sex ratio:* (*>1F:1M*).** Optimal growth is achieved with close to two females per adult male, whereas social constraints occur when close to parity (Knight and Emslie 2001).

- ***Proportion of calves in the population: calves aged i*) *0-3.5 years <27%, ii*) *1-3.5 years > 17% and iii*) *<1 year >8%.*** This indicator tracks recruitment of calves into the population, but does not give a measure of birth sex ratio, or of the occurrence of neonatal deaths. Additionally, unless date of birth is known through long-term monitoring, accurate ageing can be problematic for distinguishing calves of 3.5 years from sub-adults.

- ***Average annual growth rates : > 5% minimum.*** This is one of the best performance indicators, as it demonstrates the change in population size over time, and is useful for comparing different populations. A good knowledge of population size is required, which requires good monitoring. This measure is more reliable if averaged over 3-5 years to allow for uneven reproduction across years in small populations, and can allow prediction of future population trends. However, as a population measure, it does not allow understanding of why growth may be slowing, and can be retrospective.

Black rhinos have a theoretical intrinsic maximum rate of increase of around 9% (Knight and Emslie 2001; Owen-Smith 2001), or even higher (see equation 3.1 below, from (Caughley and Krebs 1983), but >5% growth is considered a minimum for established populations in good habitat (KWS 2012).

$$r_{max} = 1.5\ W^{-0.36} \text{ (weight in kg)} \qquad \textit{equation 3.1}$$

Based on a female eastern black rhinoceros, weighing approximately 1000-1200kg; r_{max} = 11.7-12.5%.

3.2.4. Relationship between performance indicators and natural growth rate across reserves

Generalised linear mixed models (GLMM's) in MLwiN version 2.02 (Rasbash et al. 2005) were used to investigate the relationship between performance indicators, population density, and population growth. Natural population growth rate was used as opposed to overall growth rate to remove the effect of net migration on population change within a given year. Parameters were calculated for each year and each reserve where records were available, and a three-year rolling average was then calculated to allow for stochasticity in population parameters such as synchronised calving. The density of rhinos within the reserve was also calculated, based on the approximate reserve size, as an indicator of how extrinsic factors may influence population growth. GLMMs allow nested random effects to be incorporated into the model (Bolker et al. 2009) to control for relatedness of data, which in this case were parameters calculated over multiple years and within multiple reserves; therefore 'year' and 'reserve' were included as random effects. Natural growth rate was used as the dependant variable, and performance indicators or density of rhinos within the reserve were then added to the model as fixed effects, to investigate their effect on population growth.

To investigate how each of these variables were related to natural growth rate, a minimal model was constructed, which contained all of the performance indicators that explained a significant proportion of the variance in natural growth rate. Each fixed effect was added to the GLMM, and non-significant terms were sequentially removed until only significant terms remained. Any fixed effects that did not contribute to this minimal model were then added back in, to assess their level of non-significance. A normal error structure was used for all models of natural growth rate, and the significance of each fixed effect was determined using the Wald statistic and chi-squared (χ^2) distribution, with alpha set to 0.05.

3.3. Results

3.3.1. Population demography

All eight populations increased in size over the monitoring period (See Appendix 3 for population trends and statistics over time), with average annual population growth rates ranging from 3.40% at Nairobi to 17.79% at Ol Pejeta (Figure 3.4 (a)). Although

the average annual population growth rate at Nairobi was below the 5% target, when the growth rate was divided into the natural rate of increase (births and deaths), compared to the rate of increase due to net migration (imports and exports), the natural growth rate at Nairobi was in fact above this target, at 6.93% (Figure 3.4 (b)). The overall growth of this population appears lower due to the translocation of rhinos to establish or supplement other reserves. Furthermore, although the overall growth at Ol Pejeta has been the highest across all the reserves, 12.62% was due to importation, with 20 imports between 1989 and 1993, and 30 imports between 2007 and 2010; whereas the average natural growth over this period was 5.17%.

Figure 3.4: Average annual growth rate across the eight reserves; (a) overall growth of the populations per annum; and (b) separated into natural increase (change in population size due to births and deaths), and net migration (change due to imports and exports). Dashed line at 5% represents the minimum growth target, and error bars represent standard error of the mean across years.

3.3.2. Simulated population projection

Results from the MATLAB population projection models are given in Table 3.2, including the deterministic and stochastic growth rates (λ) for each reserve. The deterministic growth rate is based on average vital rates, and represents the projected growth per annum of a population under constant environmental conditions. Stochastic growth rates incorporate observed variance in vital rates and are obtained from computer simulations of the population projected 100 years into the future, over 1000 iterations. These predictions (mean and standard deviation, SD) reflect the natural growth rate of the population, based on demographic parameters calculated from either the last 10-year period, or the entire period where records were available.

The eight populations were all projected to grow at a rate of between 2.26% and 7.04% per annum. However, the two time-frames used for calculating input parameters for the simulated projections reflect differences in the projected growth rates (Figure 3.5). Of the seven reserves where this comparison was made (excluding Mugie established in 2004), the growth rate projected from the last 10 years data was lower in three of the reserves, Masai Mara, Nairobi and Ngulia, indicating that annual growth may have slowed over the later decade. Based on these predictions (excluding Mugie which was closed in 2012), only the Masai Mara population is unlikely to achieve the target growth of 5% per annum. The four other reserves, Lewa, Nakuru, Ol Jogi and Ol Pejeta, are all projected to grow at a higher rate based on the later 10-year data, and all four of these populations, along with Nairobi and Ngulia, are all projected to grow above the target of 5% per annum.

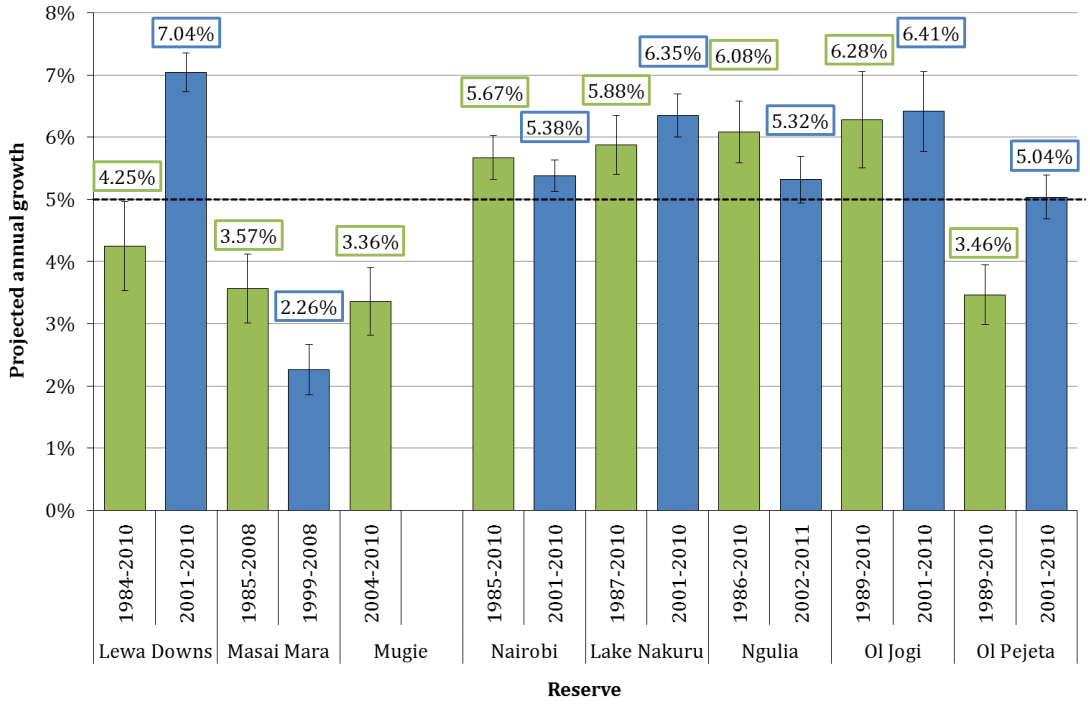


Figure 3.5: Projected annual growth rate (%) across the eight reserves from simulations run using two different time scales where applicable, based on the entire data period available (green) or the later 10 year period (blue). Dashed line at 5% represents the minimum growth target, and error bars represent standard deviation of simulated growth rates, based on 1000 iterations.

Table 3.2: Projected population growth rates for eight populations of eastern black rhinoceros in Kenyan reserves, based on vital rates calculated from all available data, or from the latest 10-year period. Deterministic growth rate (λ) represents growth of a population under constant environmental conditions, whereas stochastic growth rates (and standard deviation, SD) incorporate observed variance in vital rates.

Reserve	Time-frame	Deterministic λ	Stochastic λ (mean)	Stochastic λ (SD)	Projected annual growth rate
Lewa Downs	1984-2010	1.0440	1.0425	0.0072	4.25%
	2001-2010	1.0701	1.0704	0.0031	7.04%
Masai Mara	1985-2008	1.0380	1.0357	0.0056	3.57%
	1999-2008	1.0226	1.0226	0.0040	2.26%
Mugie	2004-2010	1.0361	1.0336	0.0054	3.36%
Nairobi	1985-2010	1.0576	1.0567	0.0035	5.67%
	2001-2010	1.0541	1.0538	0.0026	5.38%
Lake Nakuru	1987-2010	1.0591	1.0588	0.0047	5.88%
	2001-2010	1.0640	1.0635	0.0035	6.35%
Ngulia	1986-2008	1.0628	1.0608	0.0050	6.08%
	1999-2008	1.0547	1.0532	0.0037	5.32%
Ol Jogi	1989-2010	1.0644	1.0628	0.0077	6.28%
	2001-2010	1.0654	1.0641	0.0064	6.41%
Ol Pejeta	1989-2010	1.0345	1.0346	0.0048	3.46%
	2001-2010	1.0508	1.0504	0.0035	5.04%

3.3.3. Demographic performance indicators across reserves

Performance indicators calculated for each reserve, two potential time-frames, and compared to the pre-defined targets are summarised in Table 3.3.

3.3.3.1. Mortality and population structure

Generally average mortality rates across populations and age-categories were well below the targets set for optimal growth, and in most cases, below 1-2% per annum. However there were a number of incidences of relatively high mortality, for example at Lewa in 2009, presumably due to severe drought (Anon 2009) resulting in high first-year mortality, at Ol Jogi in 2010 due to a disease outbreak (Ndeereh et al. 2012), and at Mugie where two calves born within 6-months of their mothers' translocation died within 30 days of birth.

In terms of population structure, the majority of reserves achieved the optimal sex-ratio target of greater than 1 female per male, with the exception of Nakuru, where the ratio was close to parity (0.99). With the exception of Lewa, Ol Jogi (1989-2010) and Nakuru (2001-2010) the proportion of calves in the population was generally below the optimal target of >8% calves aged 0-1 and >28% calves aged 0-4, presumably related to the under-performance on a number of reproductive parameters.

Table 3.3: Mean performance indicators calculated for each reserve over the specified time-frames, compared against the pre-defined targets to achieve minimum 5% annual growth (du Toit et al. 2001), as established at the SADC rhino management group (RMG) workshop on biological management to meet continental and national black rhino conservation goals (Emslie 2001a). Indicators in green are where reserves have on average achieved targets across the monitoring period; indicators in red have on average failed to achieve the optimal targets for that performance indicator; indicators in black are where no benchmark has been established.

Average Demographic Parameter	Target	Lewa 1984-2010	Lewa 2001-2010	Mara 1984-2008	Mara 1999-2008	Mugie 2004-2011	Nairobi 1985-2010	Nairobi 2001-2010	Nakuru 1987-2010	Nakuru 2001-2010	Ngulia 1986-2008	Ngulia 1999-2008	Ol Jogi 1989-2010	Ol Jogi 2001-2010	Ol Pejeta 1989-2010	Ol Pejeta 2001-2010
Females:																
Age at first reproduction	<7 years	7y 9m 9d	7y 0m 25d	10y 0m 20d	10y 7m 9d	7y 0m 20d	8y 7m 20d	8y 3m 21d	6y 10m 28d	6y 1m 26d	8y 7m 21d	10y 8m 14d	7y 9m 24d	7y 10m 13d	8y 4m 3d	8y 8m 15d
Inter-birth interval	<3 years	2y 8m 14d	2y 5m 25d	3y 1m 2d	3y 1m 2d	2y 7m 4d	3y 2m 21d	3y 0m 18d	3y 2m 25d	2y 11m 7d	4y 0m 2d	4y 3m 15d	2y 9m 11d	2y 10m 10d	3y 2m 6d	3y 0m 2d
Adult females [a] breeding per annum	>30%	28.74%	32.75%	15.76%	12.02%	22.29%	25.10%	26.32%	25.40%	27.07%	17.78%	17.97%	30.19%	23.59%	25.14%	25.58%
Annual population mortality rate [b]	<4%	1.71%	0.99%	0.63%	0.62%	2.00%	0.55%	0.81%	0.78%	0.99%	0.05%	0.08%	1.60%	2.42%	1.87%	1.86%
Annual infant [c] mortality rate	<10%	1.31%	3.21%	3.40%	0.00%	14.29%	0.00%	0.00%	0.00%	2.98%	0.00%	0.00%	3.72%	0.00%	0.00%	0.00%
Annual sub-adult [d] mortality rate	<5%	0.68%	1.69%	0.00%	0.00%	0.00%	0.23%	0.60%	0.00%	0.00%	0.19%	0.42%	1.19%	2.50%	3.01%	0.72%
Males:																
Age at first reproduction	-	12y 0m 24d	-	14y 7m 27d	14y 7m 27d	6y 6m 5d	10y 5m 26d	10y 5m 26d	10ys 3m 4d	10ys 3m 4d	8y 7m 31d	-	11y 3m 18d	13y 1m 13d	-	-
Adult males [a] siring per annum	-	38.4%	46.9%	28.21%	12.62%	17.56%	31.03%	31.02%	24.77%	29.73%	26.55%	29.87%	57.00%	22.06%	27.1%	29.04%
Annual population mortality rate [b]	<4%	3.34%	1.63%	0.69%	0.63%	2.96%	0.56%	0.39%	0.68%	0.69%	1.11%	1.64%	1.47%	1.36%	2.24%	2.20%
Annual infant [c] mortality rate	<10%	10.87%	17.00%	0.00%	0.00%	10.98%	0.84%	2.18%	0.00%	0.00%	0.00%	0.00%	6.43%	4.15%	5.88%	0.00%
Annual sub-adult [d] mortality rate	<5%	4.71%	1.98%	0.83%	0.00%	0.00%	0.56%	1.79%	0.37%	0.57%	0.00%	0.00%	2.07%	3.04%	2.50%	1.67%
Adult sex ratio (#F:1M)	>1	2.38	1.94	1.66	1.04	1.08	1.27	1.24	0.99	1.12	1.48	1.67	2.10	1.36	1.10	1.11
Proportion of calves 0-4 years	>28%	29.62%	34.36%	20.95%	15.93%	24.02%	24.78%	27.54%	25.36%	30.60%	23.75%	19.75%	30.97%	27.06%	23.61%	27.54%
Proportion of calves <1 year old	>8%	8.88%	9.25%	5.89%	4.01%	6.64%	7.33%	7.52%	7.68%	7.85%	6.74%	5.17%	9.02%	6.76%	7.42%	7.67%
Average annual growth rate	>5%	8.57%	7.94%	5.23%	3.45%	5.11%	3.40%	3.14%	6.57%	3.11%	13.01%	6.66%	7.97%	8.19%	17.79%	11.92%
(Natural growth rate)		5.95%	8.91%	5.23%	3.45%	6.26%	6.93%	6.79%	7.75%	7.47%	7.68%	6.66%	8.43%	5.45%	5.17%	6.34%
(Migration rate)		2.62%	-0.97%	0.00%	0.00%	-1.15%	-3.54%	-3.65%	-1.17%	-4.36%	5.33%	0.00%	-0.46%	2.74%	12.62%	5.58%

[a] adult females age 5-32; adult males age 7-32; [b] average mortality of population as a whole, not separated by age class or category; [c] infant refers to calves age 0-1; [d] sub-adult females are age 1-5; sub-adult males are 1-7; [e] benchmarks use calves 0-3.5, but this is difficult to distinguish in practice, and for simplicity 0-4 has been used with data as yearly census.

3.3.3.2. Reproductive parameters

Average age at first reproduction in females was generally above the optimal target of 7 years old, except for at Nakuru. In this reserve, females on average began breeding earlier at around the age of 6-7 years. Where this indicator could be estimated, 61% of females achieved this target. However, as can be seen in Figure 3.6(a), the variation in age at first birth was high both within and across reserves, with known dams starting to breed from 4 years 7 months to 18 years of age. Furthermore, no other reserve achieved this target in more than 50% of females; Ol Pejeta being the closest, with 42% of females breeding before age 7 (Table 3.4). However, as only births to known dams could be used to calculate these data, there is a possibility that if a female's first calf was not assigned to a dam, and therefore a female may have only been recorded with a subsequent calf, age at first birth could potentially be over-estimated.

Although no target has yet been established, average age at first conception for males is higher than age at first birth for females (Figure 3.6(b)), ranging from around 6 years of age, to 17 years. Published estimates of sexual maturity are around 7-10 years (Garnier et al. 2001), but this is often based upon acquiring a territory and being able to defend a potential mate against rival males, as opposed to physiological sexual maturity. The data compiled here indicates that similar to other reports (Garnier et al. 2001), males may be able to breed earlier, if given the opportunity. Similarly to the females above, there were a high proportion of unknown sires in the dataset, which could potentially over-estimate age at first conception, if males had bred but not been recorded. However, despite the number of unknown dams and sires, it is clear that a high degree of inter-individual variation exists in the age at which they first reproduce.

Similarly, there is high variation in the length of inter-birth intervals (Figure 3.7), with three out of the eight reserves achieving the target of less than 3 years on average (Lewa 73%, Mugie 71% and Ol Jogi 69% of all intervals <3years). Additionally, 65% of intervals at Nakuru between 2001 and 2010 were also less than 3 years. At Mara, Nairobi and Ol Pejeta, although the average IBI was just over 3 years, more than 50% of intervals calculated were below this target (Table 3.4),whereas at Ngulia the average IBI was higher, at around 4 years. Again, some caution should be used when interpreting these data however, particularly with a view to the outliers and extreme outliers marked on Figure 3.7, as calves born to unknown dams could not be used in this calculation, leaving the possibility that females with an IBI of 6 years or more could

have produced a calf without it being correctly assigned. Similarly, IBI could be over-estimated if neonatal deaths were not detected or recorded.

The average percentage of adult females breeding per annum was also sub-optimal across reserves over the study period. With the exception of Lewa (32.8% 2001-2010) and Ol Jogi (30.19% 1989-2010), reserves were not meeting the target of at least 30% females breeding each year (Table 3.4). As can be seen in Figure 3.8 (a), again there was wide variation in the percentage of females breeding each year, both within and between reserves. Lewa was the only reserve where this target was achieved during more than half of the years recorded, and in contrast, both Mara and Ngulia failed to meet this target in more than three-quarters of the monitoring period. However, with the exception of these two reserves, average percentage of females breeding exceeded 20%. The percentage of males siring was also highly variable (Figure 3.8 (b)), but again, no benchmark has yet been established for this parameter.

Table 3.4: Percentage of occasions where female reproduction performance indicator targets were achieved across the monitoring period at eight Kenyan reserves.

	Lewa 1984-2010	**Mara 1984-2008**	**Mugie 2004-2011**	**Nairobi 1985-2010**	**Nakuru 1987-2010**	**Ngulia 1986-2008**	**Ol Jogi 1989-2010**	**Ol Pejeta 1989-2010**
Age at first reproduction:<7years								
Total number of females where age at first birth could be estimated	21	16	6	25	23	15	7	12
% females achieving target	38.1%	12.5%	33.3%	32.0%	60.9%	20.0%	14.3%	41.7%
% females not achieving target	61.9%	87.5%	66.7%	68.0%	39.1%	80.0%	85.7%	58.3%
Inter-birth interval :<3years								
Total number of subsequent calf pairs where intervals could be estimated	60	41	7	96	67	30	36	29
% intervals achieving target	73.3%	61.0%	71.4%	52.1%	52.2%	40.0%	69.4%	55.2%
% intervals not achieving target	26.7%	39.0%	28.6%	47.9%	47.8%	60.0%	30.6%	44.8%
Adult females breeding:>30% per annum								
Total number of years where parameter could be estimated	27	25	8	26	24	23	22	21
% years achieving target	55.6%	12.0%	37.5%	26.9%	37.5%	21.7%	31.8%	38.1%
% years not achieving target	44.4%	88.0%	62.5%	73.1%	62.5%	78.3%	68.2%	61.9%

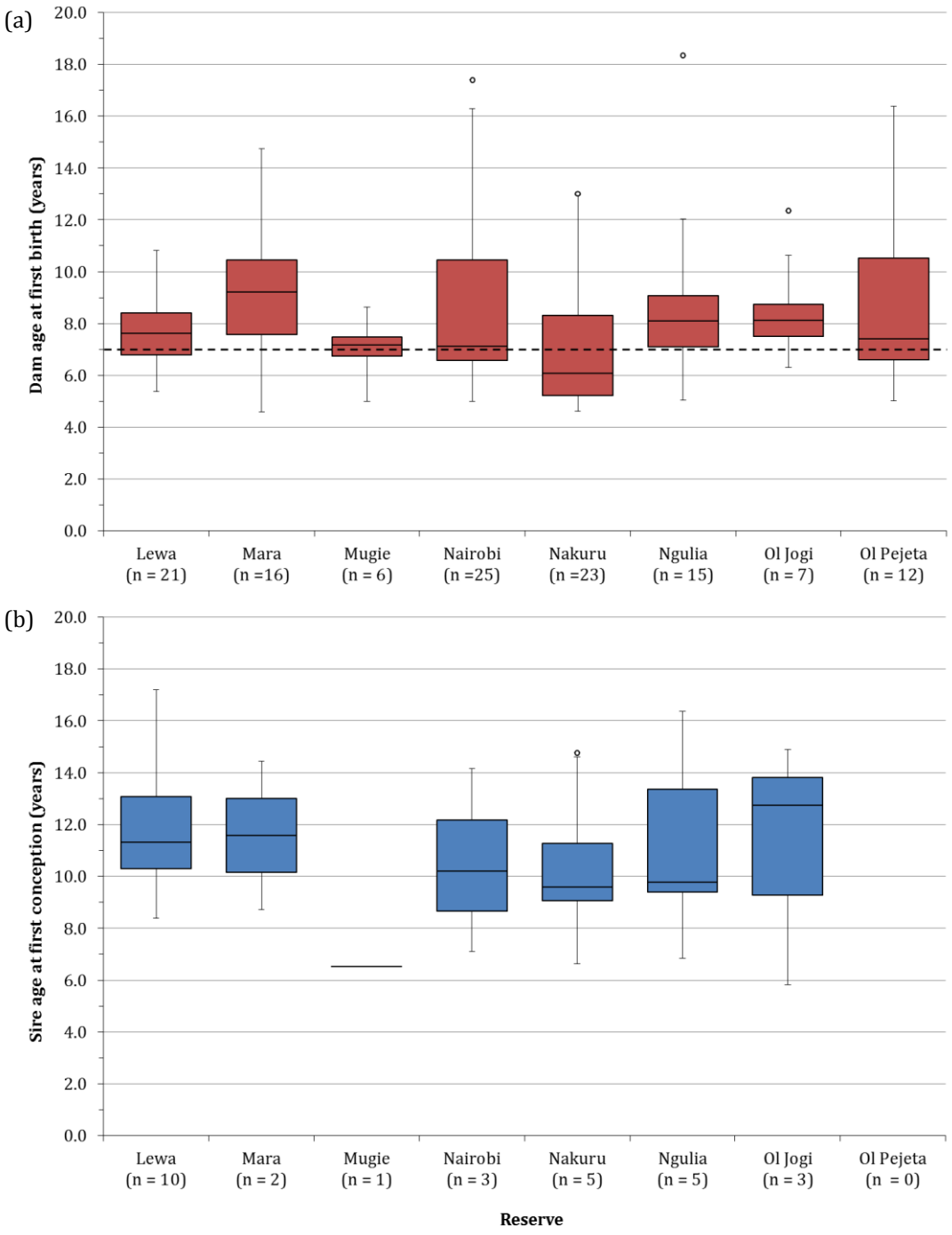


Figure 3.6: Boxplot of age at first birth for known dams (a), and age at estimated conception for known sires (estimated as 450 days prior to birth) (b) in each of the eight Kenyan reserves. The shaded box represents the interquartile range (IQR; 25th and 75th percentile of the data); the line through the box represents the median value; lower and upper whiskers represent the minimum and either the maximum value or 1.5x IQR respectively; hollow dots represent outliers (>1.5x IQR). As not all dam and sire identities were known, numbers in brackets are the number of known individuals from which parameters could be estimated. The dashed line at 7 years represents the ideal maximum age at first birth for females, as proposed for optimal population growth; there is no dashed line representing the ideal maximum age at first conception for males, as none has yet been proposed.

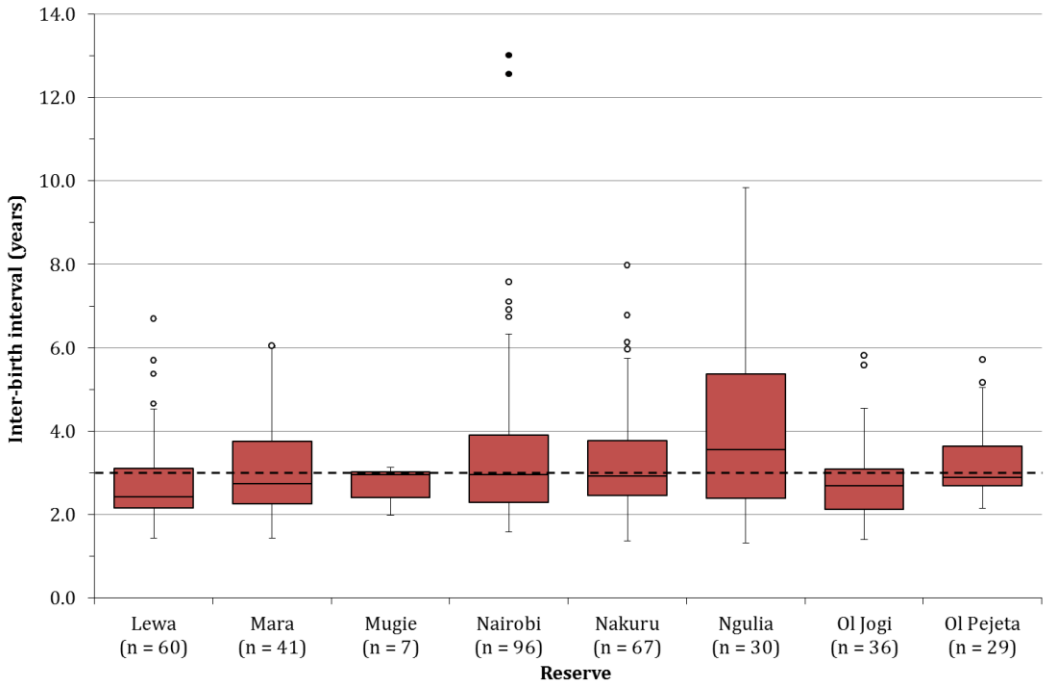


Figure 3.7: Boxplot of inter-birth interval for known dams with more than one calf in each of the eight Kenyan reserves. The shaded box represents the interquartile range (IQR; 25th and 75th percentile of the data); the line through the box represents the median value; lower whiskers represent the minimum value; upper whiskers represent either the maximum or 1.5x IQR; hollow dots represent outliers (>1.5x IQR) and closed dots represent extreme outliers (>3x IQR). Dams are only included once they have had two calves, and may be included more than once, if three or more calves have been born. The numbers in brackets are the number of subsequent calf pairs from known dams which could be used to estimated inter-birth interval. The dashed line at 3 years represents the optimal inter-birth interval, above which may indicate sub-optimal performance.

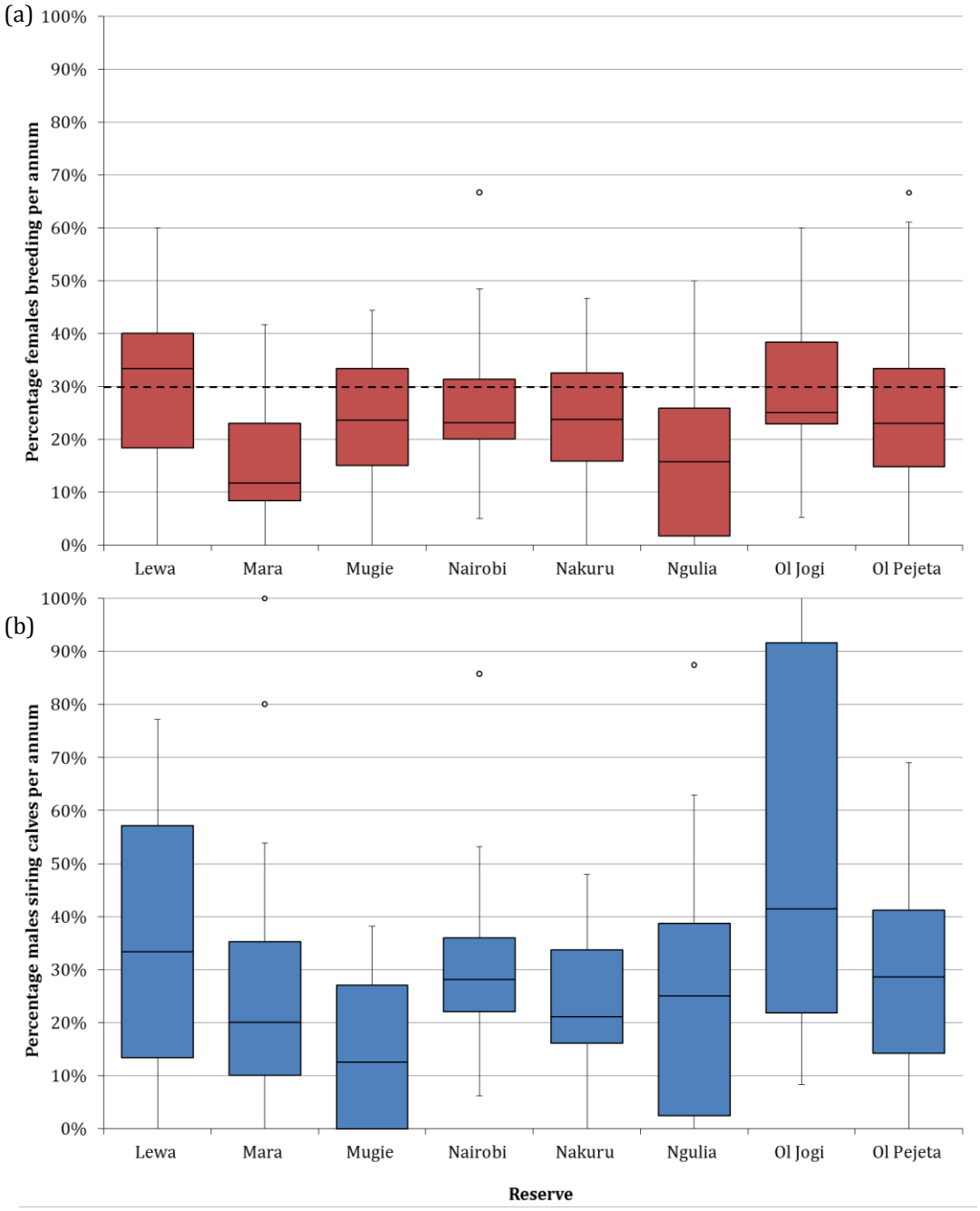


Figure 3.8: Boxplot of percentage of (a) females and (b) males breeding per annum in each of the eight Kenyan reserves. The shaded box represents the interquartile range (IQR; 25th and 75th percentile of the data); the line through the box represents the median value; lower and upper whiskers represent the minimum and either the maximum value or 1.5x IQR respectively; hollow dots represent outliers (>1.5x IQR). As individual females cannot produce more than a single calf within a particular year, all births were included, regardless of whether dam's identity was known. The dashed line at 30% represents the minimum percentage females breeding each year, as proposed for optimal population growth. As the identity of the sire is not always known, any births that could not be assigned to a sire were counted as a new sire, potentially over-estimating the number of actual sires in a given year. There is no dashed line, as the minimum percentage males breeding each has not yet been proposed.

To determine whether the variation observed in reproductive performance indicators might be due to differences over time, reproductive parameters were compared between original wild-caught founder females, and females that were born within reserves. Although the exact origin was not known for every female, females were considered to be wild-caught if their origin was unknown but they were born prior to 1985, and those that were transferred from another reserve were considered to be 'reserve-born' regardless of whether their dam was known, as it is relatively uncommon for females to be re-translocated (Benson Okita-Ouma, *pers. comm.*). According to these criteria, out of a total of 256 females aged 5-35 during the recording period, 41 were considered to be wild-caught and 215 were considered to be reserve-born.

Firstly, the number of calves born to females for each year spent in the reproductive age-class (5-35) was compared. When all females were included (whether having produced offspring or not), wild-caught females produced more calves per year in the reproductive age-class than reserve-born females (Median test χ^2=5.692, df=1, P=0.017), Figure 3.9 (a)). However, when including only females that have been recorded as having produced at least one calf (non-breeding females excluded), there was no difference between wild-caught and reserve-born females, although there was a non-significant tendency for reserve-born females to produce more calves per year in the reproductive age-class (Median test χ^2=1.202, df=1, P=0.273; Figure 3.9 (b))).

Secondly, age at first reproduction and inter-birth interval were compared between wild-caught and reserve-born females. There were no differences observed in either age at first reproduction (Median test χ^2=2.926, df=1, P=0.087), or inter-birth interval (Median test χ^2=2.237, df=1, P=0.135) between females of different origins. The difference in reproductive output between the two groups appears to be the number of reserve-born females that have not yet bred; compared to only 2/41 wild-caught females age 5-35 that died before breeding (4.88%), there were 7/215 reserve-born females age 5-35 that died without breeding (3.26%) and 79/215 reserve-born females age 5-35 are still alive but have not yet bred (36.74 %), of which 39 are age 5-9 (18.14%), 25 are age 9-17 (11.63%) and 15 are age 17-33 (6.98%).

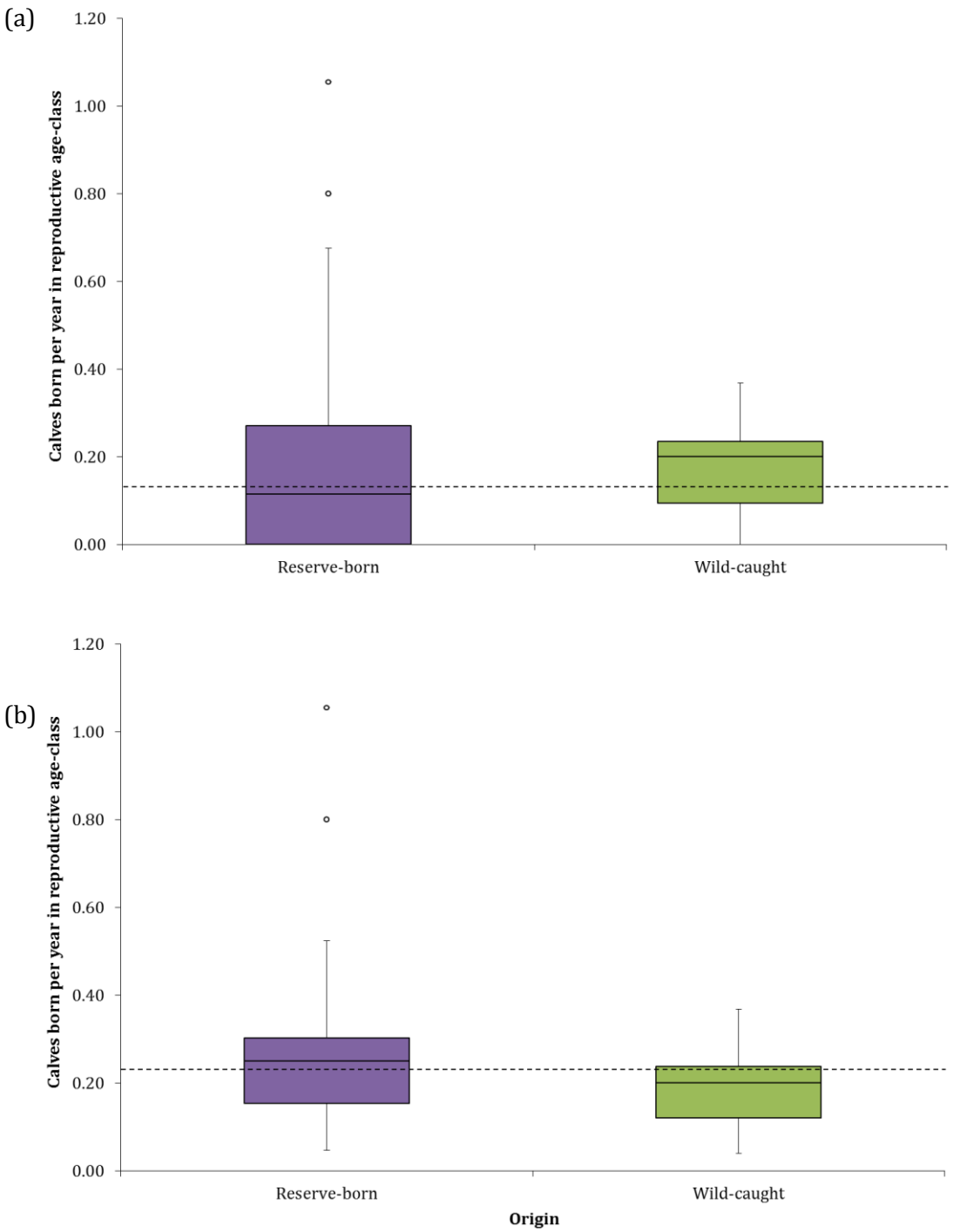


Figure 3.9: Boxplot of number of calves born per year in the reproductive age-class, comparing wild-caught and reserve-born females; (a) all females and (b) only females that have been recorded as producing a calf. The shaded box represents the interquartile range (IQR; 25th and 75th percentile of the data); the line through the box represents the median value; lower and upper whiskers represent the minimum and either the maximum value or 1.5x IQR respectively; hollow dots represent outliers (>1.5x IQR); the dashed line represents the grand mean of the two groups.

3.3.4. Performance indicators and natural growth rate across reserves

The relationship between performance indicators and natural growth rate across reserves are given in Table 3.5. Natural growth rate is determined by the change in population size due to the number of births and deaths in a population; as expected, natural growth rate was higher when a higher proportion of adult females were breeding each year, when the proportion of calves in the population was higher, and when both male and female mortality was lower. Interestingly, natural growth rate was also higher across reserves when population density was lower, and adult sex ratio was skewed towards more females in the population. These performance indicators are good predictors of natural population growth rate, and therefore can prove useful in assessing population performance.

However, it is also likely that many of these parameters may be correlated with one another, explaining similar portions of the observed variance in natural growth rate. This could impact the results of these analyses, and could potentially explain why no relationship was observed between female age at first reproduction and natural growth rate, and why the relationship with inter-birth interval is actually contrary to that expected, with higher natural growth rate when IBI is slightly longer. The inconsistent relationships between these performance indicators and natural growth rate might also be related to the high degree of variation observed in these parameters, and the impact that births to unknown dams could have an influence on these measures. There was however a relationship between male age at first reproduction, with higher natural growth rates when males sire their first calf at a younger age. Additionally, natural growth rate was also higher when a lower proportion of males were siring each year. Again, this sounds counterintuitive, but may be a population size effect, as when population size is small, a higher proportion of adult males may be siring offspring, perhaps even a single dominant male, but actually producing fewer calves than in a larger population where a lower proportion of adult males may be siring in a given year, but producing multiple calves.

Table 3.5: Factors affecting natural population growth rate, across eight Kenyan reserves, using 3-year rolling averages, and controlling for repeated measures of parameters calculated across multiple years and multiple reserves. Each parameter was entered into a single GLMM to assess the combined effect on natural growth rate, using random effects to control for year and reserve. Any non-significant terms were sequentially removed to reach the minimal model, and then re-entered to obtain their level of non-significance. The table shows the direction of the relationship, parameter estimates (effect), standard errors (SE), statistical values (Wald statistic) and significance evaluated against a χ^2 distribution (P) based on 177 annual parameters taken across eight Kenyan reserves.

	Relationship	**Effect Size (SE)**	**Wald statistic**	**df**	**P**
Significant terms in minimal model:					
Male mortality rate	Negative	-0.613 (0.046)	176.852	1	**<0.001**
Female mortality rate	Negative	-0.686 (0.084)	88.893	1	**<0.001**
Proportion adult females breeding per annum	Positive	0.216 (0.025)	76.170	1	**<0.001**
Proportion of calves <1 year old	Positive	0.612 (0.085)	51.992	1	**<0.001**
Population Density (rhinos per km^2)	Negative	-0.035 (0.006)	34.210	1	**<0.001**
Adult sex ratio (#F:1M)	Positive	0.012(0.002)	24.339	1	**<0.001**
Proportion adult males siring per annum	Negative	-0.047 (0.013)	13.732	1	**0.001**
Male age at first reproduction	Negative	-0.003 (0.001)	18.898	1	**<0.001**
Inter-birth interval	Positive	0.020 (0.004)	20.827	1	**<0.001**
Non-significant terms:					
Female age at first reproduction	None	0.002 (0.002)	1.152	1	0.28

3.4. Discussion

As with other rhinoceros species globally, Kenyan populations of eastern black rhinoceros (*Diceros bicornis michaeli*) were decimated by poaching during the 1970's and 1980's, reaching a low of around 400 individuals. Since the early 1990's, numbers have been steadily increasing, thanks to conservation efforts and the intensive management of remaining populations. On-going biological management of black rhino populations is essential to achieve target growth rates of at least 5% per annum, and to maintain genetic diversity to maximise future viability. In order to manage these populations effectively, it is important to understand how demographic parameters influence population performance, to ensure that high rates of growth are maintained.

Of the eight populations included in this study, all have increased in size since the beginning of the monitoring period, and simulated population projections predict natural growth rates based on historical demographic data of between 2.26% and 7.04% per annum. However, the populations at Masai Mara and Mugie were not predicted to reach the optimal target of 5% growth per annum. Newly established reserves are generally not expected to achieve this target (KWS 2012), and with this in mind, Mugie had been a relatively successful population, with average annual growth rates over the initial 5-year period of 9.9%. However, due to the increased threat of poaching in the area, the reserve had to be decommissioned as a rhino sanctuary in 2012 (KWS 2012). The Masai Mara population on the other hand was a well-established population, but the reduced growth rates could potentially be linked to habitat effects.

The Masai Mara is the only free-ranging population included in this study, and such populations often fail to meet the 5% target (KWS 2012). One theory for the reduced growth rates recorded for this population could be related to the migration of rhinos out of the reserve into neighbouring areas (Walpole and Bett 1998; Walpole 2002), particularly across the border into Tanzania, making monitoring of individuals more difficult to achieve. Alternatively, habitat degradation and limited suitable browse availability could be limiting growth within this reserve (Okita-Ouma et al. 2007). In Ngulia, the problem of habitat degradation has already been the focus of active management, including the removal of competing herbivores from the reserve (Okita-Ouma et al. 2008). This may have explained the slowed rate of growth during the 10 year period from 1999-2008.

In order to understand what factors may influence population performance, a number of indicators have been developed to assess whether populations are likely to achieve optimal growth rates of at least 5% per annum (du Toit et al. 2001; du Toit 2001; Knight and Emslie 2001). These indicators can be useful to population management, providing information on population dynamics and individual performance, potentially providing early warning signs to indicate when changes need to be made. These indicators provide measures of performance based on more than one aspect of demography, including mortality, population structure and reproduction.

Applying these performance indicators to the reserves in this study indicates that none of these populations appear to be achieving the specified targets on all parameters, but are growing close to or exceeding the 5% target annual growth rate. In general, mortality targets are being achieved across reserves, with the exception of particularly bad years, either resulting from drought (Lewa 2009), disease (Ol Jogi 2010), or potentially as a consequence of pregnant females translocated to a new reserve (Mugie 2004/5). With the exception of these extreme events, the number of neonatal deaths recorded was quite low, although there is a possibility that there could be some births and early deaths going undetected or unrecorded. If this were the case, this could potentially also impact on reproductive performance indicators such as age at first birth and inter-birth intervals, where performance was often sub-optimal. Similarly, any mid-term pregnancy losses would likely go undetected, but could also increase the interval between successful calves and age at first birth. The percentage of adult females breeding each year was also generally below the target of 30%, and the proportion of calves in the population was also slightly sub-optimal across reserves. However, with most reserves maintaining an adult sex ratio skewed towards females, growth rates tended to be on target.

However, it is clear from the estimates summarised in this study that these performance indicators can be quite variable both between individuals, and across years. It is therefore important to consider not only average parameters, but also the amount of variation that is acceptable within a population. In particular, reproductive parameters calculated from these data have been quite variable over time, with average age at first reproduction, inter-birth interval and percentage females breeding per year often failing to reach the specified targets needed to achieve 5% growth. However, this does not appear to be a difference between females that were wild-caught and those that have been born within reserves, but instead may reflect

individual differences. It is not yet clear whether these differences may be intrinsic, or could potentially be the result of extrinsic factors such as density and local population structure (Hrabar and du Toit 2005; Okita-Ouma et al. 2010; Patton et al. 2008), or habitat quality (Okita-Ouma et al. 2008); areas of particular interest for further investigation.

This highlights the importance of long-term monitoring, not only at a population level, but also of sub-populations within reserves, and on an individual basis to gain a better understanding of why success may vary. Furthermore, none of the indicators used here give any representation of the proportion of individuals failing to reproduce or only producing a single calf during their lifetime. Although reproductive skew in males is a normal occurrence in this species (Garnier et al. 2001), the proportion of females failing to reproduce is species such as this has yet to be fully addressed. However, due to the finite size of remaining populations, the number of both males and females breeding should ideally be maximised to maintain genetic diversity within reserves.

Although a proportion of the females in each reserve are achieving the specified targets, not all females are performing equally. This results in higher average age at first birth, longer average inter-birth intervals and a lower proportion of females breeding each year. These parameters have been calculated over a time-frame of between 8 and 27 years, and some error may be expected due to unrecorded dam identities in some cases, or the potential for unrecorded deaths within these populations. However, that many of the reserves appear to have not consistently met the reproductive parameter targets may mean that there is still room for improvement. If we could better understand what intrinsic and extrinsic factors may affect these parameters, growth could potentially be increased further, which would be beneficial for the black rhino conservation rhino programme as a whole.

Despite the variation observed in some of these indicators, 3-year rolling averages calculated for each of the performance indicators were good predictors of natural growth rate across populations. In particular, mortality, population structure and measures of overall reproductive output such as the percentage of adults breeding and proportion of calves in the population were strong predictors of growth. Individual statistics such as inter-birth interval and age at first reproduction were less useful at predicting overall population growth, perhaps due to the variation that exists between individuals. Again this highlights the need to incorporate both population performance, and individual performance indicators, to gain an overview of the demographic and

genetic health of populations. However, some caution should be taken with interpreting the relative strength of these performance indicators, due to the potential issue of correlation between these variables. This could explain why individual measures seem to be slightly less useful predictors, if the variance in growth was already explained by correlated terms within the model. Similarly, this could explain the perhaps counterintuitive positive relationship observed between inter-birth intervals and population growth rate.

Furthermore, rhino density was also negatively correlated with natural growth rate. This highlights the importance of the biological management that is already a key aspect of *in situ* black rhino management. Black rhinos are ideally managed at the maximum productivity carry capacity (Adcock 2001; KWS 2012), which is generally around 75% of the ecological carrying capacity, to maintain rapid population growth within these reserves. This illustrates that monitoring of population performance is vital, so that changes can be made before population growth begins to decline, to ensure overall rates of growth remain high.

Although every effort was made to ensure the accuracy of demographic data used in this study, the possibility remains that some data may not have been recorded. In particular, the possibility that individual deaths may not have been recorded if a carcass was never found. If this were the case, this could have an impact upon both mortality (and therefore survival) rates, and also on other demographic rates if the number of individuals at risk in a given age category may have been affected. This could therefore impact upon predictions made and ultimately on population management based on these data. This highlights the importance of good quality demographic monitoring, including a measure of the level of confidence in records obtained, for example how recently an individual has been observed (Brett 1993; du Toit 1989). This factor is of particular relevance in the data presented here from the Masai Mara, where one of the potential confounds could be the dispersal of individuals across the park boundary into Tanzania (Walpole and Bett 1998; Walpole 2002), so there is a possibility that some individuals may have died, but deaths may not necessarily have been recorded.

This study provides a thorough review of demographic information across multiple reserves, to estimate important performance indicators and population growth rates. This information can be a useful tool to improve our understanding of individual and population responses to social and environmental factors, which may impact upon

overall performance. Ultimately, a better understanding of performance indicators *in situ* will also help us understand the biology of the species for optimal biological management. Furthermore, this information can also help manage both newly established *in situ* populations and *ex situ* populations, by providing a measure of what this species can achieve under managed natural conditions, and act as a target to which other populations can aim towards to maximise growth rates and overall viability. In Chapter 4 of this thesis, this information will be used as a reference for the *ex situ* population, to evaluate population performance and investigate where there may be potential improvement.

3.5. Conclusion

- Demographic data were compiled from eastern black rhinos in eight Kenyan reserves, to assess population growth rates and performance indicators.
- All eight reserves had positive rates of growth over the monitoring period; with simulated population projections also predicting varying degrees of positive growth.
- In four of seven reserves where multiple time-frames were used for analysis, simulations using demographic data over the last decade produced higher growth rate projections, indicating that perhaps growth may have been increasing over the last decade.
- However, two reserves were not predicted to meet the annual growth rate target of 5% per annum.
- Performance indicators were calculated for each reserve, and on average, mortality and population structure indicators were achieving targets established for optimal growth.
- However, average reproduction indicators often failed to meet these targets, with a high degree of variation between individuals possibly resulting in sub-optimal population measures.
- Furthermore, although performance indicators were good predictors of natural population growth rate, due to this variation individual measures such as age at first reproduction and inter-birth intervals may be better suited to assessing individual, rather than overall population performance.

- To maximise the future viability of populations, measures of individual success are important, to recognise what proportion of the population are not currently reproducing, or are consistently failing to reach optimum targets.
- This study has provided a thorough assessment of demographic parameters across reserves, providing information on demographic parameters and population performance that can be a useful tool for guiding management both *in situ* and *ex situ* (Chapter 4).

3.6. Acknowledgements

The work in this chapter would not have been possible without the contributions of the following people, to whom I am very grateful.

- Benson Okita-Ouma, Cedric Khayale and Antony Wandera from Kenya Wildlife Service, for providing demographic information on KWS managed rhino populations for use in Chapters 3 and 4.
- Amy Dunham for technical advice on population viability analysis

CHAPTER 4

4. MAXIMISING THE CONSERVATION POTENTIAL OF THE EUROPEAN CAPTIVE POPULATION OF EASTERN BLACK RHINOCEROS: USING DEMOGRAPHIC PARAMETERS AND POPULATION VIABILITY ANALYSIS TO UNDERSTAND POPULATION PERFORMANCE.

Summary

Ex situ populations play an important role in the conservation of endangered species, acting as a vital reserve should reintroduction or supplementation become required, as well as enhancing public awareness of global conservation issues. However, in order to fulfil these roles, captive populations must be sustainable, and should be managed scientifically and cooperatively, to ensure their long-term viability.

The aim of this chapter was to determine whether the European population of black rhinoceros is self-sustainable in the long term, and determine factors that influence population performance. Demographic information from the European Association of Zoos and Aquariums (EAZA) studbook of the eastern black rhinoceros were used to calculate age-specific fecundity and mortality rates from the last 25 years, and stochastic simulations were run to predict the likely future growth rate of the population. The relative impacts of survival and reproduction on population growth were assessed, and demographic parameters from this captive population were analysed over two different time-frames, and additionally compared with data from managed *in-situ* populations in Kenya (Chapter 3) to assess where potential for improvement may exist.

The European captive population is currently self-sustaining, but is under-performing compared to its *in situ* counterparts, only growing at a rate of 1-2% per annum, compared to the target of 5%, and the average growth per annum *in situ* of 7.47%. For this population to be sustainable in the long-term, a higher rate of reproduction is required, and founder representation could be improved, by reducing the proportion of non-reproductive individuals in the population.

4.1. Introduction

Ex situ populations can play an important role in global conservation strategies (Baker 2007; IUCN 2002; WAZA 2005), acting as a vital reserve to safeguard endangered species against extinction, whilst allowing propagation in captivity should reintroduction or supplementation become required. Species such as the Arabian oryx (*Oryx leucoryx;* (Stanley-Price 1989)), black footed ferret (*Mustela nigripes;* (Jachowski and Lockhart 2009; Vargas et al. 1998)), California condor (*Gymnogyps californianus;* (Walters et al. 2010)), Przewalski's horse (*Equus ferus przewalskii;* (Ryder 1993)), and red wolf (*Canis rufus;* (Phillips et al. 2003)), were all considered to be extinct in the wild, but are now conservation success stories, following successful captive breeding and reintroduction programs. Flagship species in zoos and aquaria also act as important ambassadors to educate, and increase both public awareness (Moss and Esson 2013) and financial support for global conservation issues (Gusset and Dick 2011). Furthermore, maintaining species in captivity allows research into species biology and optimal husbandry practices, which can be of benefit to captive breeding programs, and information gained can also be applied *in situ* (Redford et al. 2012; Seddon et al. 2007).

However, to fulfil these goals, captive populations must be self-sustaining, often without supplementation from the wild (Lees and Wilcken 2009, 2011), and populations must be managed scientifically, and cooperatively, to ensure their long-term viability (Foose 1980; Foose and Wiese 2006; Leus et al. 2011b). To support conservation efforts, coordinated captive breeding programs including the European Endangered Species Breeding Program (EEP) in Europe and the Species Survival Plan (SSP) and Population Management Plan (PMP) in America have been established for a wide variety of species (Baker 2007). These coordinated programs mean that although individuals may be physically separated at multiple institutions, they can be managed as a single population, thereby increasing the potential sustainability of captive breeding programs. One particular concern of cooperative breeding programs is to maintain healthy, demographically sustainable populations with sufficient genetic diversity for future viability (Lacy 2013).

Captive breeding programs are often limited both in terms of the number of founders, and total population size (Lacy 2013). However, to act as a viable reserve for endangered species, it is important that *ex situ* populations are genetically sustainable, to retain the natural characteristics representative of their *in situ* counterparts

(McPhee and Carlstead 2010) and sufficient genetic diversity for individuals to survive and reproduce, while the population maintains the potential to adapt to future changes in the environment, without becoming adapted to captivity (Frankham 2008; Williams and Hoffman 2009). A population is generally judged to be sustainable in the long-term, according to certain criteria; reproduction should at least equal mortality; populations should be demographically stable, with 95-99% probability of population survival over a given time period; and genetic diversity should be maintained above 90% for 100 years (Amin et al. 2006; Foose et al. 1995; WAZA 2005).

However, more recently, it has been suggested that many cooperative breeding programs are failing to reach these targets (Conway 2011; Leus et al. 2011a; Long et al. 2011), and these initial criteria may not be sufficiently strict to preserve the viability of *ex situ* populations in the longer term (Lacy 2013). An initial founder population of 20 individuals can be sufficient to achieve the specified 90% genetic diversity for 100 years (Lacy 1989; Soule et al. 1986). However, this calculation was based on effective population size, and in reality, founder contribution is often uneven, meaning that a minimum of 30-50 founders is often required to achieve an effective population of 20, and retain the necessary level of diversity (Lees and Wilcken 2009; Leus et al. 2011a). An important aspect of captive management is therefore to minimise reproductive skew, ensuring that all founders are well represented within the population (Ballou et al. 2010), to slow the rate of genetic change. For some species, it may also be necessary to manage *ex situ* populations globally, and even exchange individuals with *in situ* populations, taking a metapopulation approach (Conway 1995; Lacy 2013; Stanley-Price and Fa 2007) in order to achieve these goals.

Captive breeding programs have the potential to play a vital role in the conservation of the black rhinoceros (*Diceros bicornis*) (Emslie and Brooks 1999), both in terms of maintaining an *ex situ* population as a safeguard, but also as a potential source of surplus individuals for reintroduction where safe and practical to do so (Fyumagwa and Nyahongo 2010; Holečková 2010). Accordingly, a target growth rate of 5% per annum has been proposed by the EEP (M. Pilgrim, *personal communication*). However, captive populations of black rhinoceros in North America have not been self-sustaining (Carlstead and Brown 2005; Carlstead et al. 1999a; Carlstead et al. 1999b; Smith and Read 1992). Growth has been limited by high rates of mortality, attributed to a number of health problems exhibited in captive black rhinos (Dennis et al. 2007a), and inconsistent rates of reproduction (Foose and Wiese 2006). The European captive

population of eastern black rhinoceros (*D. b. michaeli*) currently consists of approximately 10% of the global population of this sub-species (KWS 2012), but the sustainability of this population has yet to be formally assessed.

To maximise the conservation potential of this population, it is vital to understand the factors that may influence its current and future viability. Population viability analysis (PVA) is a useful management tool in conservation biology, which uses quantitative methods to predict the likely future status of a population, and can be applied to both *in situ* (Carrete et al. 2009; Daleszczyk and Bunevich 2009) and *ex situ* populations (Faust et al. 2006; Faust et al. 2003). This approach is commonly used to assess population performance, determine whether factors such as low reproduction or high mortality may be limiting population growth, and investigate effective management strategies. Simple count-based approaches to PVA can be conducted with minimal data, but may assume that all individuals within a population will contribute equally to the future population. However, in structured populations this is often not the case, as individuals may vary in their contribution according to characteristics such as their age, size, developmental stage, or their social rank. By dividing individuals in a population into unambiguous classes based on these characteristics, structured demographic models incorporate information about how reproduction and survival varies between classes.

PVA can be used to estimate the likely future growth rate of a population (Wittmer et al. 2010), the risk of extinction over a given time period (Lee et al. 2011), or the time required to reach a target population size (Earnhardt et al. 2001). This information can be useful to guide management decisions, by quantifying the relative contribution of particular groups of individuals to overall population growth (Dunham et al. 2008; Fernandez-Olalla et al. 2012), enabling targeted management. This makes PVA an integral part of species management (Boyce 1992). Furthermore, through the on-going monitoring of demographic parameters, the factors that influence the viability of a population can be established. This allows necessary changes to be made, to achieve the goals of the population, and of the metapopulation as a whole.

The aim of this chapter was therefore to use demographic information and PVA to determine whether the European captive population of eastern black rhinoceros is demographically and genetically self-sustaining, and to investigate where changes could potentially be made to maximise population performance. Specifically, differences in demographic parameters were compared within this population over

time, and between *in situ* and *ex situ* populations of eastern black rhinoceros, to identify sources of variation in population growth and potential viability, which can be used to inform future management decisions.

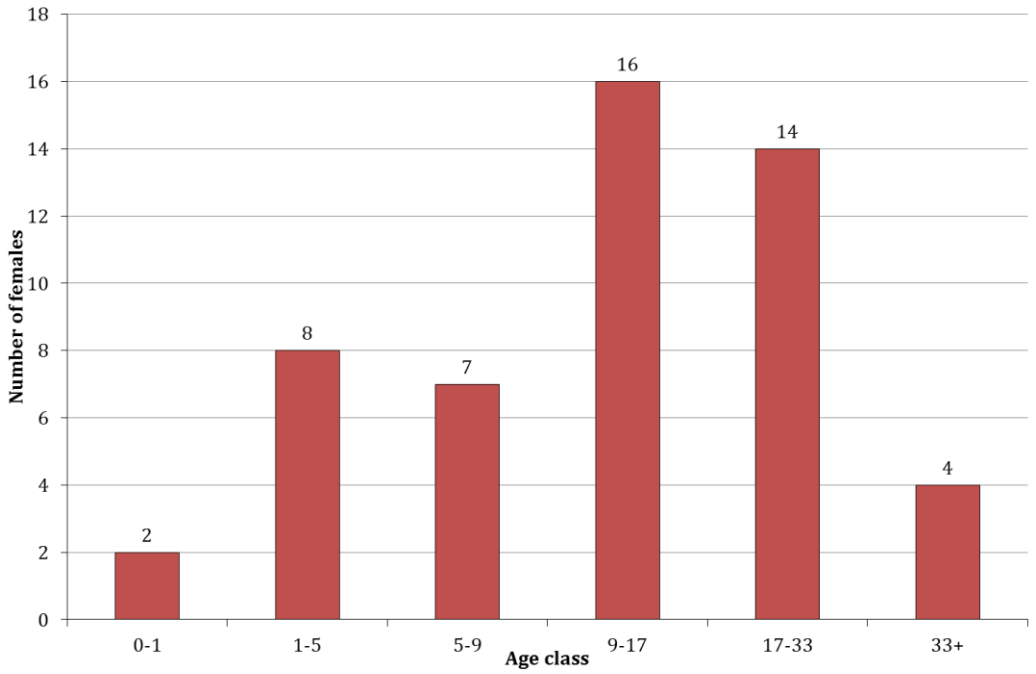
4.2. Methods

4.2.1. Demographic information

Demographic data were compiled from the European Association of Zoos and Aquariums (EAZA) studbook for the Eastern black rhinoceros (*D. b. michaeli*), contained within the Single Population Animal Record Keeping System (SPARKS; (ISIS 2004)), using a data collection window from 1st January 1986 to 31st December 2010. The population as of 31st December 2010 consisted of 78 individuals; 27 males and 51 females, situated at 15 institutions across Europe. The age structure of the population on 31st December 2010 is given in Figure 4.1; grouped into classes according to demographic characteristics.

Data were compiled on all births and deaths in the population during this time period and used to calculate age-specific fecundity and mortality rates. During the period from 1st January 2001 to 31st December 2010, growth had been noticeably slower than during the preceding 15 years (Figure 4.2); therefore to reflect any potential differences, vital rates for use in computer simulations were calculated from two time periods; 1) 1st January 1986 to 31st December 2010, and 2) 1st January 2001 to 31st December 2010.

a)



b)

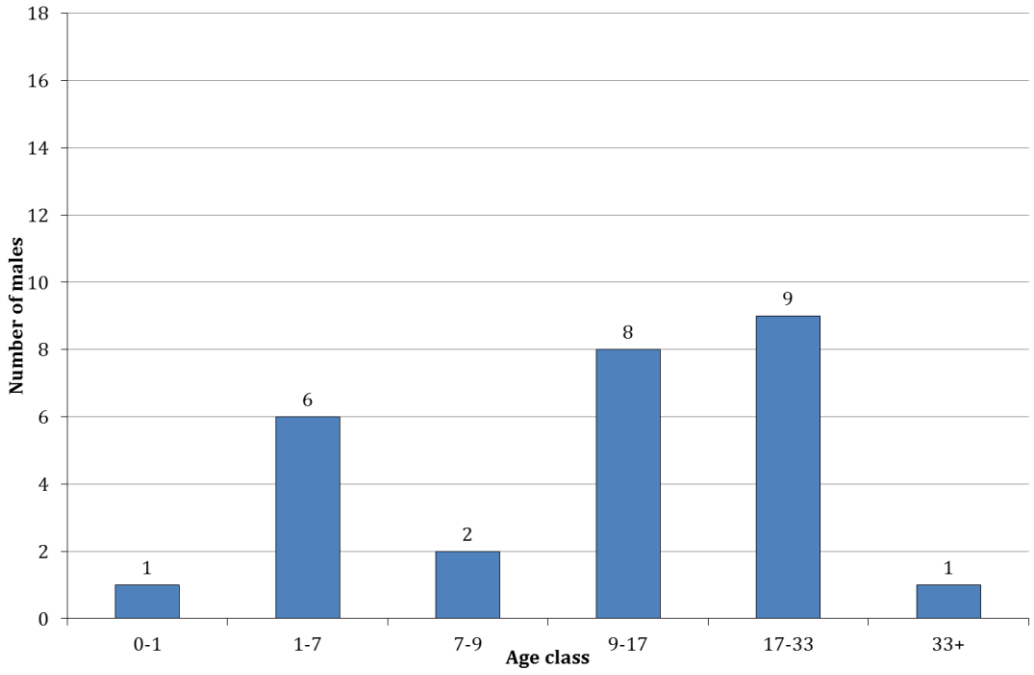


Figure 4.1: Population structure of the European captive population of Eastern black rhinoceros (*Diceros bicornis michaeli*) as of 31st December 2010; number of a) females and b) males in each of six age-classes; labels indicate the number of individuals in each class. Classes are divided into infant (0-1), pre-reproductive (1-5 for females and 1-7 for males), early reproductive (5-9 for females and 7-9 for males), 9-17, 17-33 and post-reproductive (33+).

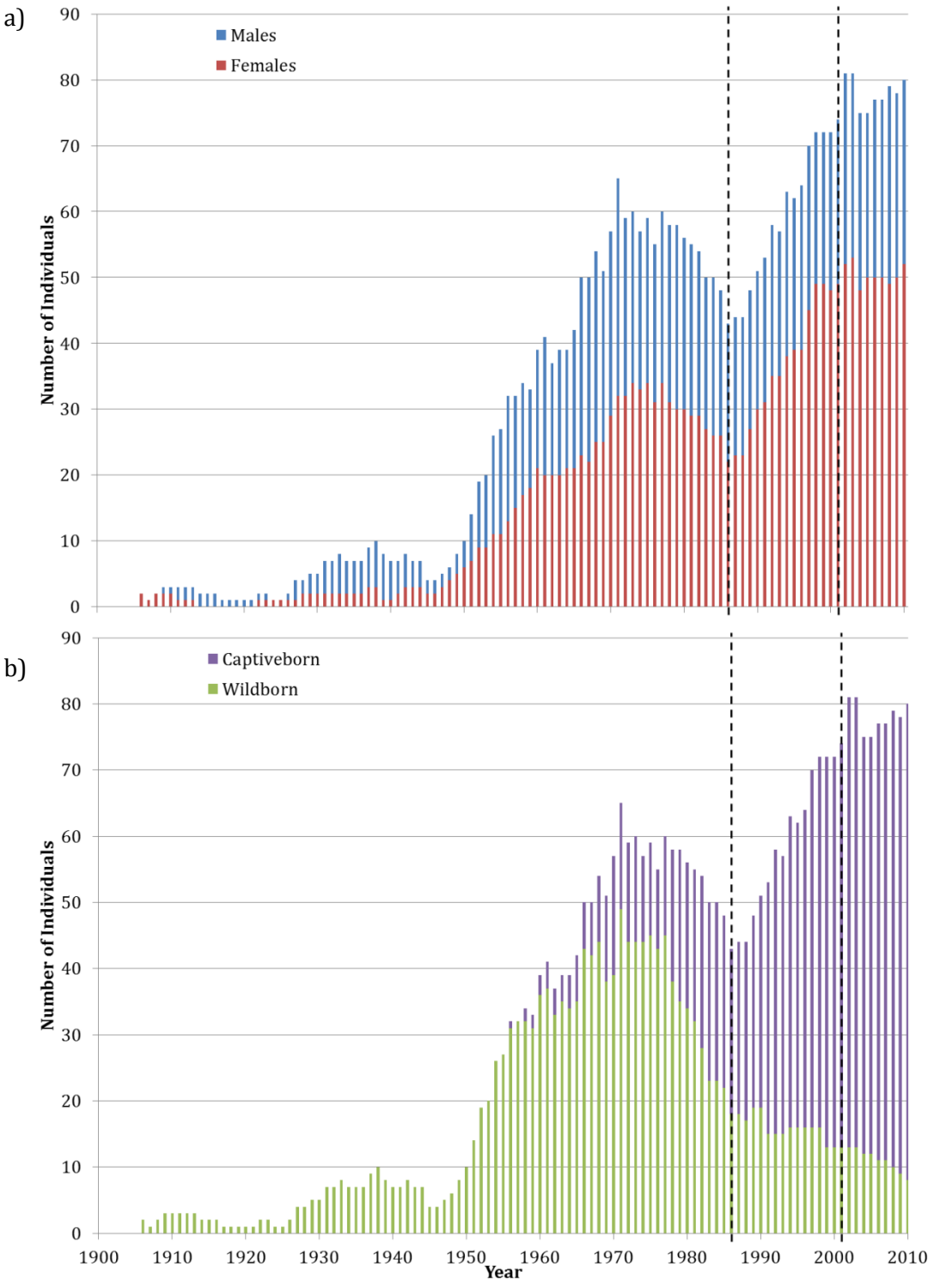


Figure 4.2: European captive population of black rhinoceros (*Diceros bicornis*), from 1900 to 2010. Column height represents the total population size, which is composed of a) number of males (■) and females (■), and b) wild-born (■) and captive-born (■) individuals. Dashed lines reflect the two time periods for data collection; 1) 1st January 1986 to 31st December 2010 and 2) 1st January 2001 to 31st December 2010.

4.2.2. Population viability analysis

To assess the demographic sustainability of the EEP population, PVA was carried out using a single-sex, female-based transition matrix model constructed in MATLAB (The MathWorks Inc 2008) using code adapted from Morris and Doak (2002), as described in full in Chapter 3 (section 3.2.2). In brief, the vital rates of age-specific fecundity, survival and transition probability were calculated for the six age classes (0-1, 1-5, 5-9, 9-17, 17-33 and 33+) (Table 4.1). A stochastic simulation was conducted using mean vital rates and variance calculated from raw data over the two time periods 1st January 1986 to 31st December 2010, and 1st January 2001 to 31st December 2010. Simulations were projected for 100 years into the future, and each simulation consisted of 1000 iterations to allow good representation of parameter combinations and produce a reliable estimate of future population size and growth rate. A quasi-extinction threshold of 20 individuals was used to assess extinction risk, and the simulation included a deterministic projection, to estimate growth of the population in a constant environment. Code used to perform these simulations is given in Appendix 4.

To investigate the potential impact of the different vital rates on overall population growth, a variance stabilised sensitivity analysis (VSS) was also performed, based on an arcsine square-root transformation (Link and Doherty 2002) (equation 4.1). When applied to vital rates that are probabilities, this transformation does not scale the variance in a vital rate to the size of the mean, so the absolute magnitude of a change has meaning independent of the value of the vital rate (Dunham et al. 2008). Sensitivity analysis code from Morris and Doak (Morris and Doak 2002) was modified to incorporate VSS (Appendix 4) calculated as follows, where θ is the vital rate under analysis (Link and Doherty 2002):

$$VSS = \frac{\partial \log \lambda}{\partial \left[2 \sin^{-1}(\sqrt{\theta})\right]} = \left[\frac{\sqrt{\theta}\,(1-\theta)}{\lambda}\right] \frac{\partial \lambda}{\partial \theta} \qquad \textit{equation 4.1}$$

4.2.3. Comparison of computer programs for conducting population viability analysis

To test the robustness of model predictions, the same data were also used to run simulations in three other PVA computer programs; Vortex (Lacy et al. 2005; Miller

2005), RAMAS Metapop (Akçakaya 2005)and ZooRisk (Earnhardt et al. 2008). These different programs represent two main differences commonly used in modelling population projections. Projections were based on either female-based transition matrix models (MATLAB, as described in Chapter 3, and RAMAS Metapop), or individual based models (Vortex and ZooRisk), where individuals and their theoretical offspring are tracked into the future. However, as these alternative programs are pre-constructed, certain assumptions have been made about the data, which were not always suited to this particular dataset. As the MATLAB model allowed the best control over how the simulation was conducted, this model was deemed to be the most robust, and so was chosen as the final method for use in these analyses. Basic results are reported for the other three programs as a comparison, but additional details on methods and full results using additional programs are provided in Appendix 5.

Two different time-scales for projection were also utilised, to obtain approximations of future population size in the short and long term; the first being more useful from a current population management perspective, and the second to approximate risk of extinction or population trends over the longer-term for species persistence (see Appendix 5 for full details). Finally, as the ZooRisk model was developed with zoo populations in mind, this was also used to incorporate a number of alternative scenarios in to the population projection. Firstly, the composition of a potential breeding group was specified as either one male with one female (1:1), or one male with two females (1:2), which are two common breeding management strategies currently used within the population. Secondly, the birth sex ratio (BSR) of the EEP population during the 25 year period between 1st January 1986 and 31st December 2010 was significantly different from parity (BSR=0.3853, χ^2=5.7339, df=1, P<0.05 based on 42 male and 67 female births). This ratio reflects a greater number of females calves born into this population over this time-frame, and this ratio was taken into account in simulated projections, again using the ZooRisk model.

Table 4.1: Vital rates calculated from the EAZA studbook for female eastern black rhinoceros (*Diceros bicornis michaeli*), based on two time periods for data collection 1) 1st January 1986 to 31st December 2010 and 2) 1st January 2001 to 31st December 2010. Vital rates include survival (s_{1-6}), transition (g_{1-5}) and fecundity (f_{3-5}). Individuals in age class 33+ cannot transition to a higher age class; and based on historical studbook data, only individuals age 5-32 have been known to reproduce.

		1986-2010		2001-2010	
Age class	**Vital Rate**	**Mean**	**Variance**	**Mean**	**Variance**
0-1	s_1	0.8718	0.0447	0.9054	0.0261
1-5	s_2	0.9921	0.0009	1.0000	0.0000
5-9	s_3	0.9832	0.0053	0.9941	0.0004
9-17	s_4	0.9879	0.0007	0.9769	0.0011
17-33	s_5	0.9748	0.0019	0.9775	0.0018
33+	s_6	0.9465	0.0249	0.9704	0.0024
0-1	g_1	1.0000	0.0000	1.0000	0.0000
1-5	g_2	0.2486	0.0197	0.2815	0.0084
5-9	g_3	0.2337	0.0231	0.2311	0.0098
9-17	g_4	0.1142	0.0119	0.0800	0.0056
17-33	g_5	0.0455	0.0047	0.0815	0.0898
5-9	f_3	0.0664	0.0076	0.0322	0.0009
9-17	f_4	0.1029	0.0141	0.0518	0.0026
17-33	f_5	0.0768	0.0048	0.0772	0.0048

4.2.4. Genetic analysis of the EEP studbook

Due to the finite size of captive breeding programs, effects such as random drift and inbreeding can be more pronounced (Lacy 1989), and can lead to the loss of genetic diversity over time. Smaller populations tend to lose genetic diversity at a greater rate, but it is not necessarily the total population size that is important, but rather the effective population size (N_e). The effective population size represents the size of an ideal population that would lose genetic diversity at the same rate as the population under consideration (Wright 1969), with genetic diversity lost at a rate of $1/N_e$. The effective population size is often smaller than the census population size (N), due to factors such as fluctuating population size across generations, high variance in family sizes, and an unequal sex ratio (Frankham et al. 2010). The effective population size was calculated for the current population (equation 4.2) based on the number of living males and females that have produced offspring. The ratio of N_e to N_c can then be used

to determine the proportion of the population that are actually contributing to the next generation (Lees and Wilcken 2009).

$$N_e = (4*Nm*Nf)/(Nm+Nf) \qquad \text{Equation 4.2}$$

One way in which the negative effects of small population size can be minimised in captive breeding programmes is through genetic management. Detailed pedigrees allow the on-going monitoring of the genetic health of a population, so that breeding can be managed to maximise the retention of founder genetics. Population management software, pm2000 (Lacy and Ballou 2002; Pollak et al. 2002), was used alongside SPARKS to extract population demography and pedigree information from the studbook, to investigate the genetic sustainability of the population. A number of population statistics were obtained, and used to estimate the genetic health of the current population based on known pedigrees. Firstly, the level of genetic diversity in the current population was estimated relative to the wild population from which the founders came, and predictions made about how this diversity could be maintained in the future based on the current population and maximum potential population growth rate based on the two population projection scenarios. Secondly, the founder genome equivalent (Lacy 1989) was calculated, which indicates how many founders would have resulted in the same genetic diversity as the current population, had they all reproduced equally.

4.2.5. Comparisons of population performance

As previously mentioned in section 4.2.1 and illustrated in Figure 4.2, growth rate of the *ex situ* population has varied over the last 25 years. To try to understand what may have led to this change in growth rate, demographic parameters were compared across the two specified time scales (1st January 1986 to 31st December 2010 and 1st January 2001 to 31st December 2010). Additionally, comparisons were also made to an *in situ* reference population, to assess how the *ex situ* population may be performing relative to their *in situ* counterparts. Firstly, a set of performance indicators for black rhinoceros as previously established to evaluate the relative success of different

populations (du Toit et al. 2001) (for description of indicators, see Chapter 3, section 3.2.3) were used to assess the performance of the EEP population. Secondly, data compiled from eight managed populations in Kenya (Chapter 3, section 3.2.1; Table 3.1) were summarised and used to calculate demographic parameters comparable to the captive population, as a direct comparison between the performance of *in situ* and *ex situ* populations over a similar timescale.

4.3. Results

4.3.1. Population performance over the last 25 years

In the EEP population of eastern black rhinoceros during the period from 1st January 1986 to 31st December 2010, there was a total of 104 calves (38 males, 63 females, and 3 of unknown sex) born to 46 dams. Unlike the SSP population of black rhinos, where a male biased birth sex ratio skew has been observed (Dennis et al. 2007b; Foose and Wiese 2006; Roth 2006), in the EEP population there was a significant birth sex ratio skew in the opposite direction, with 62.4% female and 37.6% male calves born (P=0.017). Additionally, there were 68 deaths (31 males, 34 females, and 3 of unknown sex), 11 imports from outside the EEP, and 14 exports, resulting in an average annual growth rate of 2.19% (standard deviation (SD) 5.07%). As mentioned above, the average growth of the population had been noticeably slower between 1st January 2001 and 31st January 2010 (1.15%; SD 4.24%) compared to the preceding 15 years (2.89%; SD 5.59%).

4.3.2. Population viability analysis

Based on the simulated population projection, the European captive population of eastern black rhinoceros is demographically self-sustaining. The deterministic growth rate of the population, based on average vital rates calculated from 1986-2010 and 2001-2010, was 1.0211 and 1.0012 respectively. Incorporating observed variance in vital rates into the model to simulate stochasticity produced mean stochastic growth rates (± SD) of 1.0212 (0.0048) and 1.0052 (0.0033) over the next 100 years, representing a projected growth per annum of 2.12% and 0.52% respectively. Based

on a starting population of 51 females, this simulated growth resulted in an average final population size at 100 years of 463.21 (SD 237.14) and 89.87 (SD 29.89) females respectively. Although the deterministic projection is closer to the stochastic projection (Figure 4.3) using the data calculated from 1st January 1986 to 31st December 2010 (within 1SD of the mean) than that calculated from 1st January 2001 to 31st December 2010 (outside of 1SD from the mean), both are within with range of simulated stochastic predictions. This may be related to the greater amount of data used to calculate the average and variance in vital rates from the last 25 year period compared to the last 10 year period.

Variance-stabilised sensitivity analysis using data from the last 25 years indicates that there is more potential for increasing the growth rate of the population through increasing reproduction of females aged 9-17 (0.0264), followed by increasing neonatal survival (0.0194) and reproduction of females aged 17-33 (0.019). Using data from the last 10 years in this analysis, the potential growth could be most influenced by increasing reproduction in both 17-33 and 9-17 year old females (0.0192 and 0.0186, respectively), followed by increasing the survival of 9-17 year old females (0.013).

4.3.3. Comparison of computer programs for conducting population viability analysis

The simulated population projections obtained from the different computer programs were very similar to those obtained using the MATLAB model reported above. Based on demographic information from the last 25 years, the population was projected to grow at between 2.22% (Vortex) and 2.34% (RAMAS Metapop), compared to 2.12% from the MATLAB projection. Projections based on the last 10 year data were also very similar, and still below that based on the 25 year data (Vortex 0.60% and RAMAS Metapop 0.35%, compared to 0.52% from MATLAB). This indicates that once demographic data has been compiled over a suitable time-frame to reduce the impact of demographic stochasticity on estimates of fecundity and mortality, the models were relatively comparable, despite the differences in assumptions that had been made.

Using the ZooRisk program, population projections were also run using the same demographic data, but were modified according to breeding group composition and birth sex ratio. When simulations were restricted to a single female paired with a male at any given time (1:1), this resulted in lower projected growth rates of 0.22% or

1.38% based on 10 year and 25 year data respectively. However, if the model was specified to allow two females per male (1:2), projected growth rates were increased to 1.36% and 3.02% from the two data time-frames respectively. This indicates that if breeding groups could be managed with two females per male, then growth of the population could potentially be more than doubled.

When a reduced sex ratio was incorporated into the ZooRisk simulation (25 year data BSR=0.3853; 10 year data BSR=0.4; see Appendix 5 for details), growth was further reduced when a 1:1 breeding group was simulated (growth of 1.10% based on 25 year data and decline in population size of -0.54% based on 10 year data). However, when allowing two females per male, growth was projected at the highest rate of all scenarios used (3.94% and 2.10% based on the two time-frames respectively). This suggests that although the number of males in the population has the potential to limit growth, especially if only one female is paired with each male, if breeding groups were to consist of two females with each male, growth of the population could be increased.

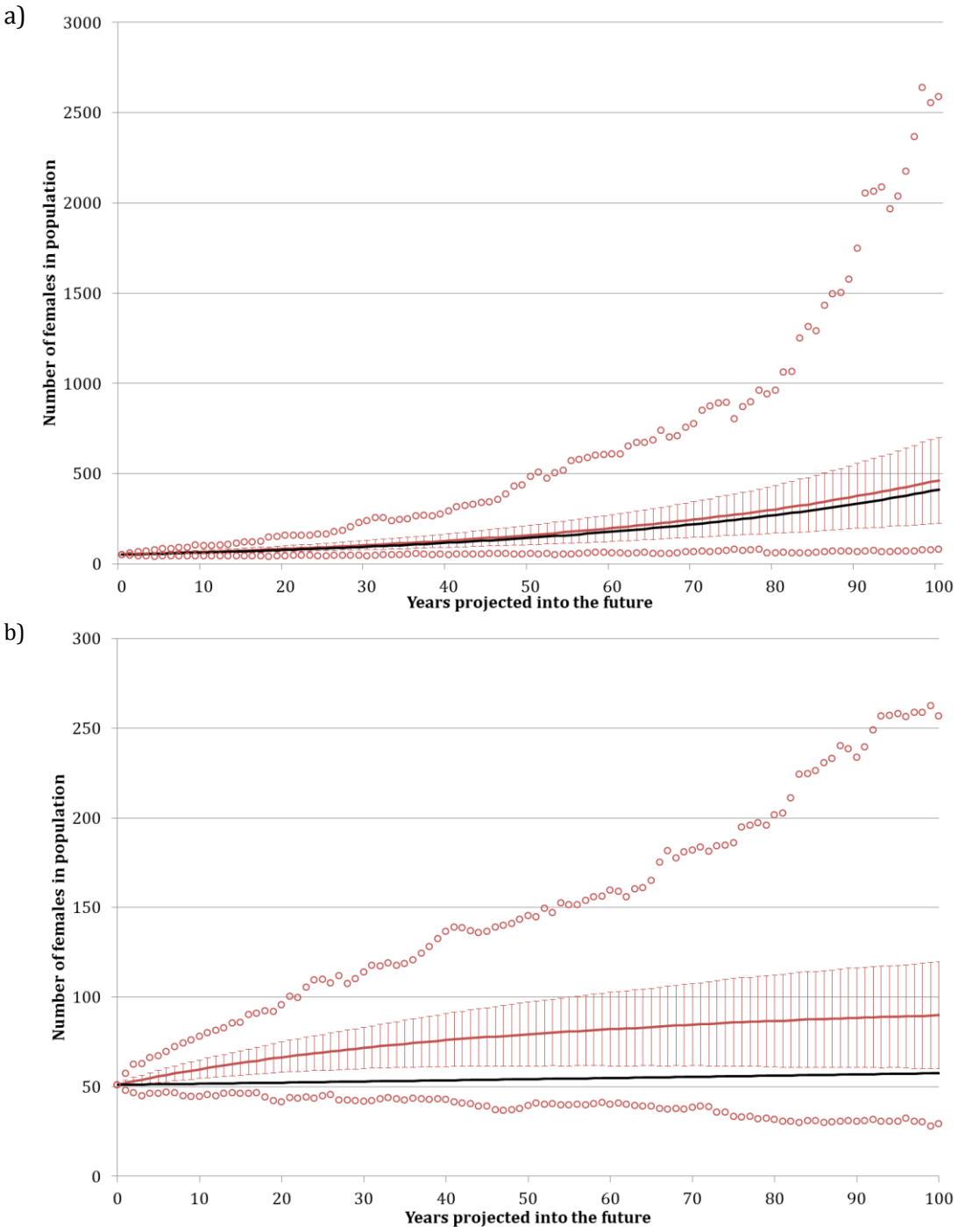


Figure 4.3: Population size (females only) projection from MATLAB model showing results from simulations using a) 25 and b) 10 year data. The average projected population size based on stochastic simulations is represented by the red line; error bars represent one standard deviation in population size across 1000 iterations; red markers represent the minimum and maximum population size estimates obtained from simulations for each year of projection. The black line represents the deterministic population projection, which is the projected growth of the population under a constant environment. Note the difference in scale on the y-axis between the two scenarios, representing the difference in final population size by 100 years.

4.3.4. Genetic analysis of EEP studbook

Of the 135 potential founders, all of which are assumed to be unrelated, and include either wild-caught black rhinos imported directly into the EEP population, or the wild-caught founders of captive born individuals imported from non-EEP populations, 41 still have living descendants. However, the contribution of these founders to the current population has been unequal, with percentage representation ranging from 0.26% to 10.24% (Figure 4.4) and 33% of the current population are related to the 5 most represented founders. This has resulted in a founder genome equivalent of 13.39 wild-caught founders had they all reproduced equally.

The effective population size (N_e) was estimated as 31.54 based on 12 male and 23 female breeders (Equation 4.2). Although this estimate will reflect the current N_e of the population, it may be an overestimate because some of the animals that have bred may no longer be reproductive, and reproduction may not be equal across the population (Lacy and Ballou 2002). Based on a census population size of 78, this gives a N_e/N ratio of 0.4044, representing around 40% of the population that are actually contributing to the next generation.

The current population has retained an estimated 96.27% of the genetic diversity (GD) of the founder population, but slow rates of growth and continued unequal representation could mean that this level of diversity may not be retained over the next 100 years. Using the two projected stochastic growth rates (as determined in section 4.3.2) based on the last 10 years and 25 years demographic data of 1.005 and 1.021, and a theoretical maximum population size of 100 individuals, deterministic projections suggest that 90% GD can only be retained for the next 88 and 93 years respectively. Even with a potentially unlimited population size, with the lower growth rate, it is likely that only 89.65% can be retained for 92 years, whereas the higher growth rate, and a theoretical growth rate of 5% per annum would require a population size of 107 and 106 individuals, respectively, to retain 90% genetic diversity over the next 100 years.

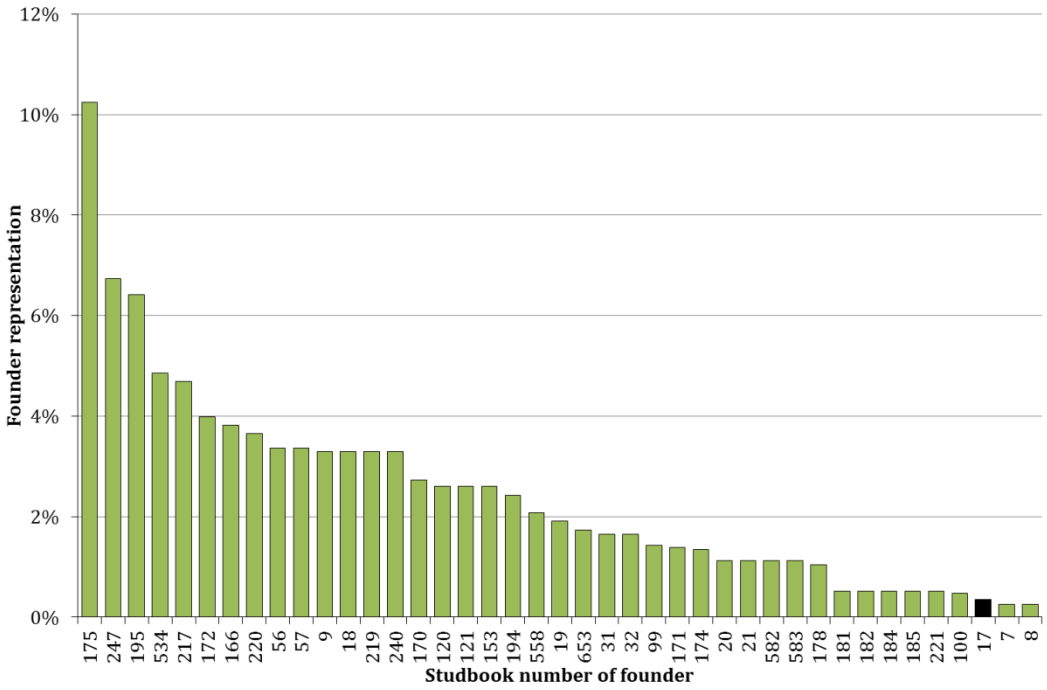


Figure 4.4: Percentage representation of the 41 wild-born founders within the current EEP population of eastern black rhinoceros (*D. b. michaeli*). Founder number 17 (black) has only a single living descendant that will not reproduce due to health problems, and so will be lost from the population.

4.3.5. Comparisons of population performance

4.3.5.1. EEP population over the last 25 years

There are marked differences between the projected population growth rates obtained from simulations using demographic data from the last 10 years compared to the last 25 years (section 4.3.2). It is therefore important to investigate what differences in the following population performance indicators may exist between these two time-frames.

4.3.5.2. Female reproduction

The vital rates calculated from these two time-frames (Table 4.1) and the predictions made using sensitivity analysis, both indicate that reproduction appears to be limiting the growth of the EEP population. Furthermore, Table 4.2 contains performance

indicators (du Toit et al. 2001) for the EEP population calculated from the two time-frames, which indicate that during the ten year period from 2001-2010, on average females were starting to breed later, with longer inter-birth intervals, and a lower proportion of females were breeding each year, compared to the 25 year time-frame. To investigate potential differences in reproduction in the EEP population over time, two non-overlapping time frames were used, which reflect periods when the average annual population growth rate was relatively high 1986-1995, and relatively low 2001-2010 (Figure 4.2).

Of the females that did breed during either of these two 10-year periods, females produced a higher average number of calves in the period between 1986-1995 (38 calves born to 18 dams 86-95 (mean 2.11), than females between 2001-2010 (37 calves born to 24 dams 01-10 (mean 1.54) (Mann Whitney U;P=0.032) (Figure 4.5).

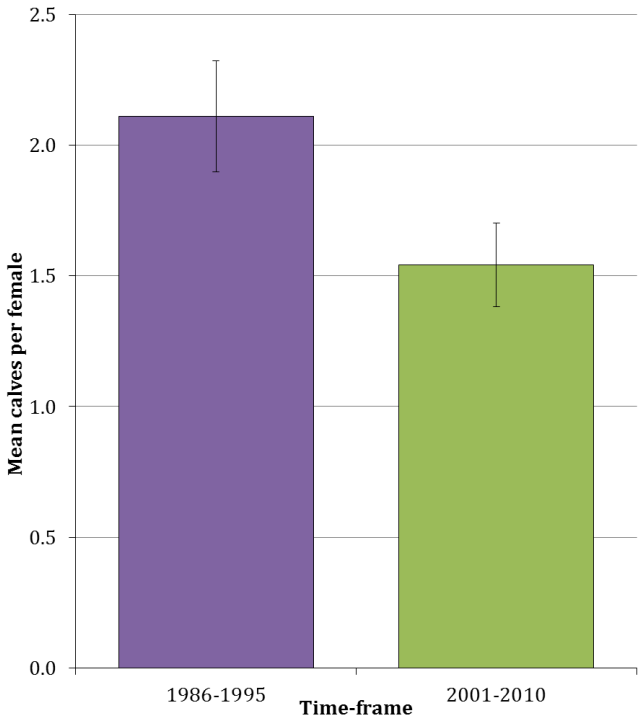


Figure 4.5: Mean number of calves (± s.e.m) per female that reproduced during the 10 year periods from 1986-1995, or 2001-2010

However, the difference in fecundity between the two periods may not just be due to individual females reproducing at a slower rate; but also due to a high degree of reproductive skew. As indicated in section 4.3.4, unequal reproduction has been an issue historically throughout the studbook. Of the 144 *D. b. michaeli* females in the EEP studbook, 52 (36.1%) produced at least one calf, whereas 64 (44.4%) either died or left the population without reproducing, and 28 (19.4%) have not reproduced, but are still alive and have the potential to contribute. This reproductive skew continues to be an issue; the current EEP population on the 31st December 2010 consisted of 51 females, 37 of which were in the reproductive age-class, but 49% had yet to produce offspring. Additionally, of the reproductive-age females that had previously produced offspring, 43.5% females had not bred for at least 5 years (mean inter-birth interval (IBI) +1SD); and 39.1% females had not bred for at least 7 years (mean IBI +2SD).

This reproductive skew amongst females could contribute towards the differences in growth rate between the two different time-frames. During the 10 year period from 1986-1995, when growth was relatively high, there were 48 females of reproductive age, of which 52.9% gave birth during that 10-year period. In comparison, during the 10-year period from 2001-2010, when population growth was slowest, there were 71 females of reproductive age; of which only 40.7% gave birth (Figure 4.6). Although there tended to be a higher proportion of non-breeding females age 5-32 in latter 10 year period, this difference was not significant across the two time periods (Mann Whitney U; P=0.255).

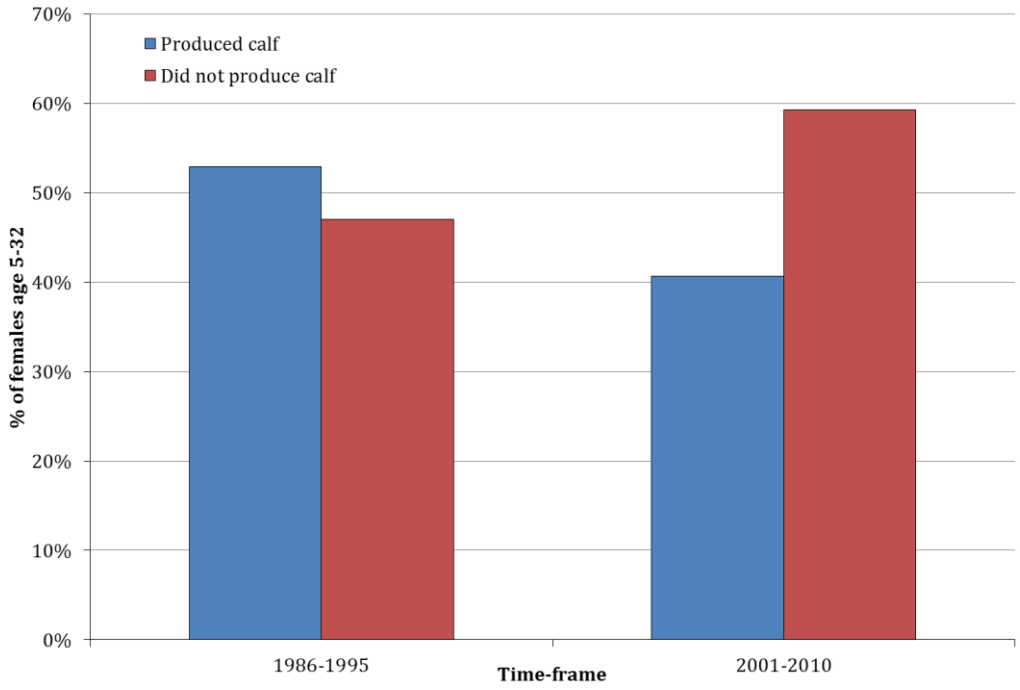


Figure 4.6: Proportion of females in the reproductive age class (age 5-32) that produced a calf, or did not produce a calf, during the 10-year periods of 1986-1995 and 2001-2010.

4.3.5.3. Male reproduction

Similarly for males, unequal reproduction appears to have been an issue, both historically, and in the current population. Out of a total of 124 *D. b. michaeli* males in the EEP studbook, 46 (37.1%) sired calves, whereas 63 (50.8%) either died or left the population without reproducing, and 15 (12.1%) have not reproduced, but are still alive. Of the 27 males in the current population, 19 are in the reproductive age class, and 42.1% of these are yet to sire offspring. Additionally, of the reproductive-age males that had previously sired offspring, 33.3% of males had not contributed for at least 5 years (mean inter-birth interval (IBI) +1SD); and 16.7 % of males for at least 7 years (mean IBI +2SD).

4.3.5.4. Mortality

Infant mortality was elevated over the last 25 years (12.87% and 22.59% for females and males respectively), but was reduced, particularly in males over the last 10 years (9.57% and 5.75%). Rates of mortality in other age classes were generally more similar between the last 25 and 10 year time-frames, and were below 2% for sub-adults (females age 1-5 and males age 1-7), and below 4% for the population overall.

4.3.5.5. Comparison to reference population

With annual growth rates in the EEP population well below the 5% target, it is important to determine where there may be potential for improvement. Average demographic parameters calculated from the *ex situ* population of eastern black rhinoceros in Europe, and the *in situ* managed population in Kenya are given in Table 4.2, alongside performance indicators as explained in Chapter 3, section 3.2.3.

4.3.5.5.1. Female reproduction

The EEP population is not currently achieving the targets necessary for optimal growth in any of the measures of reproduction, with females starting to reproduce later, and producing calves with longer inter-birth intervals. Although the average age at first reproduction and inter-birth interval in the *in situ* populations are still higher than the optimal target, both parameters are lower (i.e. closer to the target) *in situ* than both the 25 year time-period and the 10 year time-period in the *ex situ* population. Furthermore, on average across the 25 year period, only 15.7% of females were breeding per year, compared to the target of >30%, and compared to an average of 23.7% females breeding per annum in Kenya. As a result, the percentage of calves in the population is below the desired target, both in terms of infant calves aged 0-1, and total calves age 0-3.

As highlighted in section 4.3.4.1, reproductive skew amongst females in the EEP population may be one factor potential behind the reduced reproductive output of the population, leading to limited growth. To investigate this possibility, the percentage of females in each age class that have either bred successfully, died or left the population

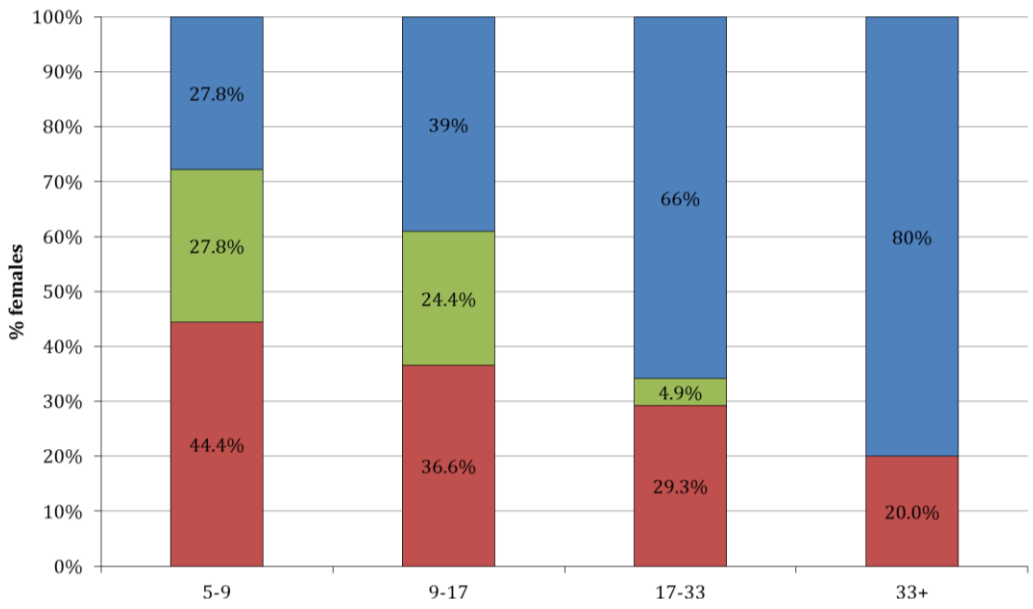
without breeding, or are still alive but have not yet reproduced, were compared to the *in situ* reference population (Figure 4.7). Firstly, using females in the current population (i.e. alive on 31/12/2010), of reproductive age (5 years and older), 49% of the *ex situ* population had yet to reproduce, compared to 37% of the *in situ* reference population. However, if only females age 9 years and older are included, to allow for the later age at first reproduction observed in both populations, 43% of the *ex situ* population had yet to reproduce, compared to only 24% of the *in situ* population. Secondly, when looking at the historical populations (i.e. including individuals that have died or left the population), the percentage of females that did not reproduce is significantly higher in the *ex situ* population compared to the *in situ* population, in all three reproductive age classes (Mann Whitney U; 5-9 ($P=0.001$), 9-17 ($P=0.003$), 17-33 ($P<0.001$)), but there is no difference in females that reached the final age class (33+ ($P=0.397$)).

Table 4.2: Average demographic parameters calculated from the EEP population of eastern black rhinoceros (*D. b. michaeli*) over the two time periods 1st January 1986 to 31st December 2010 and 1st January 2001 to 31st December 2010, compared to data from managed populations in Kenya, and against targets for population success (du Toit et al. 2001; Okita-Ouma 2004). Figures in green are currently achieving targets; figures in red are failing to achieve the optimal targets for that demographic parameter; indicators in black are where no benchmark has been established.

		EEP 2001 - 2010		EEP 1986-2010		KWS	
Demographic Parameter	**Target**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
Females:							
Age at first reproduction	<7y	9y 10m 25d	3y 2m 31d	9y 7m 21d	3y 10m 25d	8y 2m 24d	2y 7m 17d
Inter-birth interval	<3y	3y 10m 17d	11m 30d	3y 5m 4d	1y 2m 30d	3y 2m 17d	1y 5m 24d
Adult females [a] breeding per year	>30%	11.30%	4.79%	15.70%	7.76%	23.74%	5.08%
Annual population mortality rate [b]	<4%	2.22%	11.29%	2.49%	12.74%	1.19%	0.67%
Annual infant [c] mortality rate	<10%	9.57%	16.23%	12.87%	21.17%	2.84%	4.88%
Annual sub-adult [d] mortality rate	<5%	0.00%	0.00%	0.79%	2.99%	0.66%	1.04%
Males:							
Age at first reproduction	-	11y 1m 7d	5y 6m 8d	10y 10m 17d	5ys 3m 30d	11y 0m 9d	3y 1m 4d
Adult males [a] siring per year	-	22.00%	9.78%	29.20%	15.18%	31.20%	11.96%
Annual population mortality rate [b]	<4%	2.80%	15.38%	3.56%	16.49%	1.61%	1.10%
Annual infant [c] mortality rate	<10%	5.75%	16.25%	22.59%	32.78%	4.37%	4.81%
Annual sub-adult [d] mortality rate	<5%	1.76%	5.6%	0.71%	3.53%	1.38%	1.63%
Adult sex ratio (#F:1M)	>1	1.92	0.08	1.81	0.27	1.50	0.51
Proportion of calves (age 0-4)	>28%	17.63%	1.96%	20.35%	5.95%	25.4%	3.3%
Proportion of calves (age 0-1)	>8%	4.60%	1.56%	5.60%	2.73%	7.4%	1.1%
Average annual growth rate	>5%	1.15%	4.24%	2.19%	5.07%	7.47%	5.16%

[a] adult females age 5-32; adult males age 7-32; [b] average mortality of population as a whole, not separated by age class or category; [c] infant refers to calves age 0-1; [d] sub-adult females are age 1-5; sub-adult males are 1-7.

a)



b)

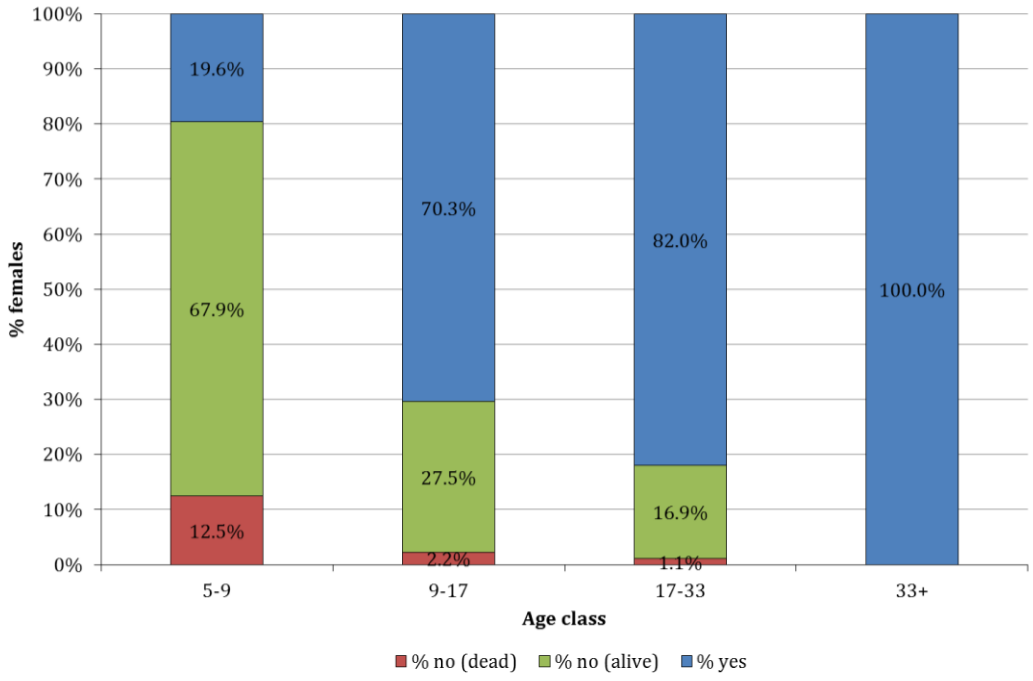


Figure 4.7: Percentage of females in each age class (5-9, 9-17, 17-33 and 33+) that produced a calf (blue), died or left the population before producing a calf (red) or have yet to produce a calf but are still alive and still have the potential to contribute (green); a) *ex situ* EEP population and b) *in situ* KWS reference population.

4.3.5.5.2. Male reproduction

Although there are no established benchmarks for male reproduction, the age at first conception is very similar across the two populations (Table 4.2). However, the percentage of adult males siring each year was slightly higher *in situ* than in the EEP population, particularly during 2001-2010.

4.3.5.5.3. Mortality

Unlike reproductive parameters, the targets for mortality are generally being achieved within the EEP population, with overall female mortality <4% across both data collection periods, and male mortality also within this target over the last 10 years. Overall male mortality was slightly elevated over the 25 years however, perhaps due to the elevated first-year mortality during this period. Female first-year mortality was also slightly higher than optimal over the last 25 years, but both male and female neonatal mortality appear reduced over the last 10 years, and were both below the target of <10%. Compared to the *in situ* population however, annual population mortality rate in the EEP population was slightly higher. The difference in mortality in the 0-1 age class should be considered with some caution however, as neonatal death may be underestimated in the *in situ* population, as stillborn calves, or those that die within the first few days of life, may not always be recorded.

4.4. Discussion

Although the EEP population of eastern black rhinoceros (*D. b. michaeli*) is currently self-sustaining, achieving demographic sustainability, and maintaining genetic diversity above 90%, it is currently performing below its potential. Growth of the population is currently below the target of 5% per annum, with PVA models predicting a future growth rate of between 0.52% and 2.12% per annum over the next 100 years. The difference in these predictions reflects the observed variation in demographic parameters and population growth over the last 25 years, which has decreased over the last decade. If this *ex situ* population is to fulfil its role, not only acting as a safeguard against further decline of the wild population, but also providing a source for reintroductions, it is essential that it grows at a sufficient rate to support any exports that may become necessary, whilst maintaining the viability of the *ex situ* population in the long term (Lacy 2013).

As indicated by the variance-stabilised sensitivity (VSS) analysis, increasing fecundity could have the greatest impact on future population growth. This is supported by the low rates of reproduction observed in this population, both in comparison to the *in situ* reference population, and in relation to the targets set for 5% growth, suggesting there may indeed be potential for improvement. Within the EEP population age-specific fecundity was lower in females aged 5-17 (age classes 5-9 and 9-17) over the last 10 years, approximately half that calculated from the last 25 years, which is likely to have contributed to the lower projected growth rate obtained. Analysis of the EEP studbook has further indicated that not only did the total proportion of females breeding decrease over the two time-frames, from 52.9% between 1986 and 1995 to 40.7% between 2001 and 2010; but those females that were breeding were on average producing fewer calves (2.11 compared to 1.54 calves per breeding female), resulting in a lower percentage of females reproducing each year (1986-2000 18.7%; 2001-2010 11.3%).This indicates that the main factor limiting growth in this population is low reproductive output, and more specifically, insufficient females reproducing each year.

This not only has the potential to impact the overall growth rate of the population, but with 42% males aged 7-32 and 49% females aged 5-32 in the EEP population yet to successfully reproduce, could also impact the effective population size (N_e) and maintenance of genetic diversity for the future. The N_e/N ratio calculated from this population based on the number of male and female breeders, was 0.4044, indicating that the effective population size is around 40% of the census population size. Although

estimates from wild populations suggest that effective population sizes are often only around 10% of the total population (Frankham et al. 2010), in captivity this proportion tends to be higher. In a meta-analysis of 40 managed captive populations (Earnhardt et al. 2004), the average N_e/N was 0.27, with values reported between 0.07 (king penguin, *Aptenodytes patagonicus* (Denton 1999)) and 0.48 (slender loris, *Loris tardigradus* (Fitch-Snyder 1999)); although values as high as 0.7 have been reported (Willis and Wiese 1993). This suggests that the N_e reported here is actually relatively high compared to other species. However, when maintaining maximum genetic diversity is a key objective in captive populations of endangered species, the reduced effective population size in this population may be limiting population growth, and the genetic potential of future generations.

As indicated by the founder genome equivalent, historical reproductive output of the population has also been unequal across individuals, with a high degree of reproductive skew within the population. This has important consequences for the captive breeding program, and the viability of the *ex situ* population in general. Reproductive skew and variance in lifetime reproductive success is not necessarily uncommon (Clutton-Brock 1988), and the loss of lineages is a natural process in wild populations, often due to differences in competitive ability, or in response to natural catastrophes (Gompper et al. 1997). However, reproductive skew in females is more commonly associated with cooperative breeders (Cant 1998), where dominant females produce the majority of offspring, and reproductive skew can be high, both within years and in lifetime reproductive success. In non-cooperative group-living species, there is often variance in reproductive success within years, based on female age (Rubenstein and Nunez 2009), but overall reproductive skew is often lower. However, in a species such as the black rhinoceros, where adult females are generally considered to be relatively solitary (Goddard 1967), the occurrence of a strong reproductive skew over time seems unusual, and the high variance in reproductive success observed in the *ex situ* population is not reflected to the same degree in the *in situ* reference population, particularly the percentage of females that died without leaving offspring (44.4% *ex situ*, 6.6% *in situ*). The current proportion of non-breeding females *in situ* is more similar (5-32 49% *ex situ* compared to 37% *in situ*; 9-32 43% *ex situ* compared to 24% *in situ*), although there may be some over-estimation *in situ* as some of the recorded births could not be attributed to known dams (57 calves born to unknown dams between 1984 and 2010). This suggests that perhaps the reproductive skew

observed in the EEP population may be an artefact of captivity, rather than a natural degree of variation.

Black rhinos naturally have a polygynous or even polygynandrous mating system (Christensen et al. 2009; Hutchins and Kreger 2006), and reproductive skew in males is more common, with dominant males tending to monopolise a high proportion of matings. Garnier et al. (2001) analysed the genetic relatedness of black rhinoceros in Zimbabwe, and found high reproductive skew amongst males; a single male sired 52.6% of all offspring during a 10 year period, and 64% of the males in the population did not sire at all. However, in captivity where founders are limited, and most institutions only hold one adult male (currently 12 out of 16 institutions in Europe only have a single mature male), unequal reproduction across males can also be problematic, automatically limiting chances of females to breed if no other potential mate is available. Historically 50.8% of males in the EEP studbook failed to leave descendants, and 42.1% of current reproductive-age males are yet to sire offspring. Furthermore, the birth sex-ratio skew apparent in this population (40:60), and the resulting adult ratio of 1:1.92, potentially limits the number of available breeding males; in fact, there are only 9 males at 5 institutions that have bred in the last 5 years, and currently 14 institutions with breeding-age females. This poses the question of how to minimise reproductive skew in captivity, to increase reproductive output and population growth rates.

As illustrated by the hypothetical population projection scenarios using the ZooRisk program, managing breeding groups as a trio of one male and two females could potentially enhance growth rates. This is a logical finding, as if an institution had such a trio, and the male successfully mated both females, two calves could be born every 2-3 years, based on an optimal inter-birth interval. However, if only one male and one female were kept, only a single calf could be born every 2-3 years. However, this is limited by both the number of mature males currently in the population, and furthermore by the large proportion that are currently non-proven. It is therefore important for the management of this population to gain a better understanding of any potential differences between individuals that could explain the differences in previous reproductive success. In Chapter 5 of this thesis, intrinsic differences in reproductive hormones between these breeding and non-breeding individuals will therefore be investigated.

Coordinated captive breeding programs can play a vital role in species conservation, both in terms of a genetic reserve, but also as a source for reintroduction into protected reserves to supplement the *in situ* population. However, the EEP population of black rhinoceros is not currently achieving its target of 5% growth per annum, limited by low reproductive output and a high proportion of individuals failing to reproduce. Within captive breeding programs, the maintenance of genetic diversity becomes a vital aspect of population management, and it is important to maximise founder genetics by equalising the contribution of lesser represented founders (Lacy 1989). A better understanding of factors influencing reproduction and differences in reproductive success in this species is essential, if the population is to reach the targets set for its performance, and the performance of its *in situ* counterparts.

4.5. Conclusion

- Although the EEP population of eastern black rhinoceros (*D. b. michaeli*) is self-sustaining, it is not currently achieving its potential.
- In particular, reproductive performance indicators are reduced compared to *in situ* populations, limiting population growth.
- Furthermore, the proportion of captive females reproducing each year has decreased in the last 10 years, compared to the last 25 year period.
- Population projections estimate future annual growth rates of between 0.5% and 2%, based on parameters calculated from the last 10 and 25 years respectively.
- If growth rates could be increased, this population could fulfil its role as a source for reintroduction and/or exchange with other captive breeding programs as part of a global metapopulation approach to conserving this species.
- However, at present, high reproductive skew, and a large proportion of the population failing to reproduce, is not only limiting the reproductive output of the population, but could have a big impact on the future genetic viability of the population.
- To maximise the viability of this population, and its contribution to conservation, a better understanding of what is limiting reproductive success is required.

4.6. Acknowledgements

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CHAPTER 5

5. INVESTIGATING INTRINSIC FACTORS ASSOCIATED WITH REPRODUCTIVE SUCCESS IN MALE AND FEMALE EASTERN BLACK RHINOCEROS.

Summary

Ex situ populations of endangered species such as the black rhinoceros can play an important role in global conservation strategies. However, at present, the European captive population is performing sub-optimally, both with respect to *in situ* populations and to specified targets for population growth (Chapters 3 and 4). Growth and future viability of this population is potentially being limited by low reproductive output and reproductive skew among both males and females, resulting in a high proportion of reproductive-age individuals currently not reproducing.

In order to improve the reproductive output of this population, the aim of this chapter was firstly to establish the current reproductive status of breeding and non-breeding individuals, and secondly to investigate intrinsic differences between individuals that may warrant further investigation. Faecal samples were collected from approximately 90% of the EEP population of eastern black rhinoceros, to establish reproductive hormone concentration in both males and females.

Faecal progesterone metabolite concentration was used to determine reproductive cyclicity among breeding and non-breeding females, and although 94% of reproductive-age females showed at least some evidence of ovarian cyclicity, periods of irregular cyclicity were common. Cycles both longer (>40d), and shorter (<20d) than the average of 27.1 (±5) days were observed, as well as periods of acyclicity. Although the overall proportion of regular compared to irregular cycles did not differ between proven and non-proven females, longer cycles were more common in non-proven females. Furthermore, within proven females, acyclicity was more common in females that had not bred during the last 7 years. In males, differences in testosterone concentration were observed, not only with increasing age, but also with higher testosterone concentration in breeding than non-breeding males.

In addition to differences in reproductive hormones, non-proven females were also assigned higher body condition scores than proven females, indicating a potential relationship between weight and reproductive success in females, but not males in this

population. Non-breeding females were also less likely to exhibit regular oestrous behaviours than proven females.

In summary, differences in reproductive hormones were observed between breeding and non-breeding males and females in this *ex situ* population, and in females, body condition and the expression of oestrus behaviours were also correlated with reproductive success.

5.1. Introduction

Although the European captive population of eastern black rhinoceros (*Diceros bicornis michaeli*) is currently self-sustaining, low rates of growth and high reproductive skew are limiting the long-term viability of this population. Historically, only 37.1% of males and 36.1% of females from the European studbook of *D. b. michaeli* have produced offspring in their lifetime, and over the last 25 years, on average only 15.7% of females breed per year. Furthermore, 42.1% of mature males and 48.6% of mature females in the current population are yet to reproduce, and a further 16.7% and 39.1% of previously proven males and females have not bred for over seven years. This has potential consequences, not only for overall growth rates, but also for the genetic health of the *ex situ* population, potentially resulting in a loss of diversity relative to the founder population and their *in situ* counterparts. It is therefore essential to investigate differences in reproductive success between individuals, to increase the overall reproductive output and optimise growth of the population.

Endocrinology has become a valuable tool in captive breeding programs, and many studies have been conducted with female black rhinoceros, both *in situ* (Brett et al. 1989; Garnier et al. 2002; MacDonald et al. 2008) and *ex situ* (Berkeley et al. 1997; Czekala and Callison 1996; Graham et al. 2001; Hindle et al. 1992; Radcliffe et al. 2001; Ramsay et al. 1987; Schwarzenberger et al. 1993; Schwarzenberger et al. 1996b), to establish basic reproductive parameters such as characterising reproductive cyclicity and pregnancy determination. However, a review of the reproductive physiology of captive black rhinos in America by Brown et al. (2001), revealed that cyclicity can be quite erratic in this species, with longer and shorter cycles than normal, as well as periods of acyclicity also observed. Although irregular cyclicity, prolonged periods of anoestrus and reproductive pathologies (Hermes et al. 2004; Hermes et al. 2006) are a common problem in captive white rhinoceros (*Ceratotherium simum simum*) (Brown et al. 2001; Carlstead and Brown 2005; Hermes et al. 2006; Metrione and Harder 2011; Patton et al. 1999; Schwarzenberger et al. 1998; Swaisgood et al. 2006), the potential causes of irregular cyclicity and possible effects on fecundity in the black rhinoceros have not yet been fully determined, nor has the prevalence of these different cycle types been investigated in the European population.

Reproductive endocrinology can also be key to understanding reproductive success in males, as androgens have the potential to influence reproduction through their effect on spermatogenesis, libido, and sexual behaviour (Roser 2008). Studies on the

reproductive endocrinology of male black rhinos have been less common than those in females (although see Brett et al. (1989); Brown et al. (2001) and Christensen et al. (2009)). No differences in testosterone concentration were observed between breeding and non-breeding males in the American captive population (Brown et al. 2001). However, variation in testosterone between individuals has been observed in captivity, and was attributed to differences in sociosexual environment (Christensen et al. 2009), with higher testosterone concentration observed in males housed with a greater number of males or females. This suggests that social factors may play an important role in testosterone concentration, and ultimately in reproductive success, although this was not addressed directly. In the white rhinoceros, testosterone concentration is higher in dominant than subordinate males, and dominant males achieve more matings (Kretzschmar et al. 2004; Rachlow et al. 1998), suggesting that higher testosterone concentration may indeed be beneficial, and there is also evidence that sperm quality and motility may also correlate with social status (Hermes et al. 2005).

In wild black rhinos, Garnier et al. (2001) observed that dominant males achieved higher reproductive success, mating with several different females and resulting in a high degree of reproductive skew. Recent research *in situ* has also indicated that reproductive success may be linked to heterozygosity, with more heterozygous males holding larger territories and siring more offspring (Cain and Watts et al., *pers. comm.*). However, in neither of these cases where differences in reproductive success were observed between males was testosterone concentration also determined. It is therefore important to investigate whether the variation in male reproductive success observed in this *ex situ* population could be related to differences in testosterone concentration. Furthermore, as both males and females in captivity are under-performing when it comes to reproduction, it is important to understand whether there are any underlying physiological differences between breeding and non-breeding individuals, either in reproductive hormones themselves, or whether other intrinsic factors could be involved.

One area that warrants investigation in *ex situ* populations is the influence of body condition on reproductive status. Research investigating reproduction in both humans and domestic animals in particular, has indicated that either too low body condition or too high body condition can potentially inhibit reproduction. For example, in dairy cattle, too low body condition has been associated with ovulatory failure, whereas too

high body condition may be related to impaired folliculogenesis, reduced oocyte quality and embryonic failure (Ferguson 2005). Similarly, in humans, under-nutrition can lead to delayed puberty and amenorrhea (Frisch and McArthur 1974), whereas obesity has been linked to a number of reproductive problems including in oocyte development, ovulation, endometrial development, implantation, embryo development, and pregnancy loss (Brewer and Balen 2010; Norman 2010). Obesity has also been linked to fertility problems in males, as excess adipose tissue increases the conversion of testosterone to oestradiol, resulting in reproductive axis suppression and reduced testosterone concentration (Michalakis et al. 2013). Furthermore oxidative stress resulting from fat accumulation has also been linked to decreased spermatogenesis (Michalakis et al. 2013). As captive diets are often over nutrient-rich compared to natural diets (Berkeley et al. 2011; Dierenfeld 1997), and particularly for the black rhinoceros which is predominantly a browsing species, natural diets may be difficult to adhere to. Subsequently, over-feeding of unsuitable food types could lead to obesity (Clauss and Hatt 2006). It is therefore necessary to take this into account when investigating factors that may influence reproductive success in this species.

Additionally, in captive black rhinoceros, breeding pairs are often kept separately, and only introduced during oestrus. Reliable detection of oestrus therefore becomes very important, to ensure opportunities for mating are provided. However, oestrous behaviours in the absence of a male are often difficult to distinguish (Fouraker and Wagener 1996), and the occurrence of overt signs can be highly variable (Radcliffe et al. 2001). Furthermore, even when a female is receptive, rhino courtship can be very aggressive; prior to full oestrus, the female will often chase the male away through mock charges and defensive displays (Hutchins and Kreger 2006). This leaves the possibility that reduced fecundity could be related to insufficient opportunities to mate, either through failure to make introductions, or by prematurely separating potential breeding pairs due to observed aggression. The occurrence and variability of oestrous behaviours in the black rhinoceros has so far received little attention, and therefore the relative occurrence of overt signs versus silent oestrus in this species is unknown. This may be one factor that could lead to reduced fecundity in this population, either through females not becoming receptive as expected, or through reduced reproductive management if overt signs are not observed by keepers. Therefore any potential differences in the expression of behavioural oestrous between breeding and non-breeding females warrant investigation.

The long term viability and sustainability of *ex situ* populations are essential for them to fulfil their purpose, and furthermore, captive breeding programs have a duty of care to provide optimal conditions. Chapters 3 and 4 of this thesis have indicated that reproduction may be lower in the EEP population of eastern black rhinoceros as compared to *in situ* populations, limiting the growth and future viability of this population. It is therefore necessary to determine whether differences between breeding and non-breeding individuals could be related to differences in reproductive hormones, and to identify intrinsic factors that may influence reproductive success in this species. There are therefore two main aims to this chapter. Firstly, to establish the current reproductive status of the EEP population of eastern black rhinoceros, using hormone analysis to determine whether and how regularly females are cycling, and to establish testosterone concentration and variability among males. Secondly, to investigate in both males and females whether intrinsic factors such as body condition, and the expression of oestrous behaviours in females could be related to reproductive success, and to explain potential differences between breeding and non-breeding individuals.

5.2. Methods

5.2.1. Study population

This study included 62 eastern black rhinos situated at 13 zoological institutions across Europe (Table 5.1), and consisted of 23 males between the ages of 2y 10m and 32y 6m and 39 females between the ages of 1y 3m and 40y 9m (Figure 5.1). This represents around 90% of the EEP population that had been at or approaching reproductive age during the study period.

The reproductive history of each individual was determined from the EAZA studbook, and individuals were categorised as follows. Firstly, individuals were categorised by their age, with females between the ages of 5-32 and males between the ages of 7-32 considered to be of breeding age; females aged under five and males under seven were classed as immature, and individuals aged 33 and over classed as being post-reproductive. Those individuals in the reproductive age class (n=17 males; n=31 females) were then further categorised as proven breeders if they had ever produced a live calf by the end of 2010 (premature births and stillbirths were not considered for this purpose, in case pregnancy loss could be a factor in poor reproductive success),

whereas those that had never produced a live calf were considered non-proven. However, to distinguish between individuals that were currently breeding and those that may have bred previously but have not reproduced for some time, a further category was established that included individuals that had not produced a calf for more than 7 years. The average inter-birth interval in this population is around 3 ½ years, so this timescale represents double the period in which a female would ideally have produced a subsequent calf. Therefore breeding age individuals were also categorised as either 1) proven breeders that had produced a calf within the last 7 years, 2) proven breeders that had not produced a calf within the last 7 years, and 3) non-proven individuals.

Table 5.1: Summary of individuals from which faecal samples were collected as part of the study, including their age and reproductive category during the period of sample collection.

SB #	Name	Location [a]	Sex	Age [b]	Breeding status [c]	Breeding status (last 7 years) [d]
384	Rosie	Chester	F	21.1	NP	NP
680	Kitani	Chester	F	13.6	P	P<7
696	Manyara	Chester	F	12.4	NP	NP
883	Zuri	Chester	F	5.0	Y	
898	Ema Elsa	Chester	F	8.2	P	P<7
947	Malindi	Chester	F	6.1	NP	NP
956	Bashira	Chester	F	3.1	Y	
532	Tisa	Doué la Fontaine	F	16.0	NP	NP
910	Binti	Doué la Fontaine	F	7.2	NP	NP
387	Jessi	Dvur Kralove	F	25.4	P	P<7
619	Elba	Dvur Kralove	F	13.7	P	P<7
685	Jola	Dvur Kralove	F	12.6	P	P<7
689	Jane Lee	Dvur Kralove	F	12.3	NP	NP
876	Maischa	Dvur Kralove	F	4.4	Y	
878	Etosha	Dvur Kralove	F	3.7	Y	
417	Sany	Hannover	F	20.5	P	P>7
436	Sabah	Hannover	F	19.1	P	P>7
754	Rufiji	Howletts	F	10.9	NP	NP
762	Salome	Howletts	F	10.3	NP	NP
437	Nane	Krefeld	F	20.7	P	P<7
295	Mana	Magdeburg	F	28.5	P	P<7
559	Maleika	Magdeburg	F	14.2	NP	NP
428	Sita	Paignton	F	21.1	P	P<7
454	Siwa	Pont Scorff	F	18.8	NP	NP
195	Rukwa	Port Lympne	F	40.8[e]	P	P>7
342	Arusha	Port Lympne	F	27.8	P	P>7
408	N'akuru	Port Lympne	F	21.5	P	P>7
455	Etna	Port Lympne	F	19.0	P	P<7
456	Jaga	Port Lympne	F	18.1	P	P>7
558	Vuyu	Port Lympne	F	19.8	P	P<7
663	Ruaha	Port Lympne	F	14.3	P	P<7
879	Zawadi	Port Lympne	F	4.3	Y	
880	Grumeti	Port Lympne	F	4.3	Y	
888	Solio	Port Lympne	F	9.7	NP	NP
911	Nyasa	Port Lympne	F	8.4	NP	NP
950	Damara	Port Lympne	F	5.3	NP	
968	Nyota	Port Lympne	F	1.3	Y	
662	Wanda	Zurich	F	14.3	NP	NP
861	Samira	Zurich	F	9.6	NP	NP
714	Magadi	Chester	M	12.8	P	P<7
750	Sammy	Chester	M	12.0	P	P<7
955	Asani	Chester	M	2.8	Y	
268	Isis	Dvur Kralove	M	32.5	P	P<7
283	Jimm	Dvur Kralove	M	31.2	P	P<7
483	Baringo II	Dvur Kralove	M	17.5	NP	NP
659	Mweru	Dvur Kralove	M	13.7	NP	NP
877	Davu	Dvur Kralove	M	4.2	Y	
926	Dzanti	Dvur Kralove	M	2.5	Y	
927	Thabo	Ebeltoft	M	4.3	Y	
928	Kito	Ebeltoft	M	4.4	Y	
349	Kifaru II	Hannover	M	27.9	P	P>7
890	Vungu	Howletts	M	8.4	NP	NP
533	Taco	Koln	M	15.5	NP	NP
528	Usoni	Krefeld	M	15.8	P	P<7
653	Madiba	Magdeburg	M	20.1	P	P<7
892	Manyara	Paignton	M	8.6	NP	NP
438	Jakob	Pont Scorff	M	19.5	NP	NP
341	Kingo	Port Lympne	M	27.5	P	P<7
430	Quinto	Port Lympne	M	20.4	P	P>7
903	Zambezi II	Port Lympne	M	8.3	NP	NP
951	Monduli	Port Lympne	M	5.3	Y	
857	Jeremy	Zurich	M	9.8	NP	NP

[a] current location when samples were collected for study; [b] age at the end of the sample collection period;
[c] P=Proven – has produced a live offspring, NP=Non-proven – has never produced a live offspring;
[d] P<7=Proven and has bred during last 7 years, P>7=Proven but not bred during last 7 years, NP=Non-proven – has never produced a live offspring; [e] estimated date of birth.

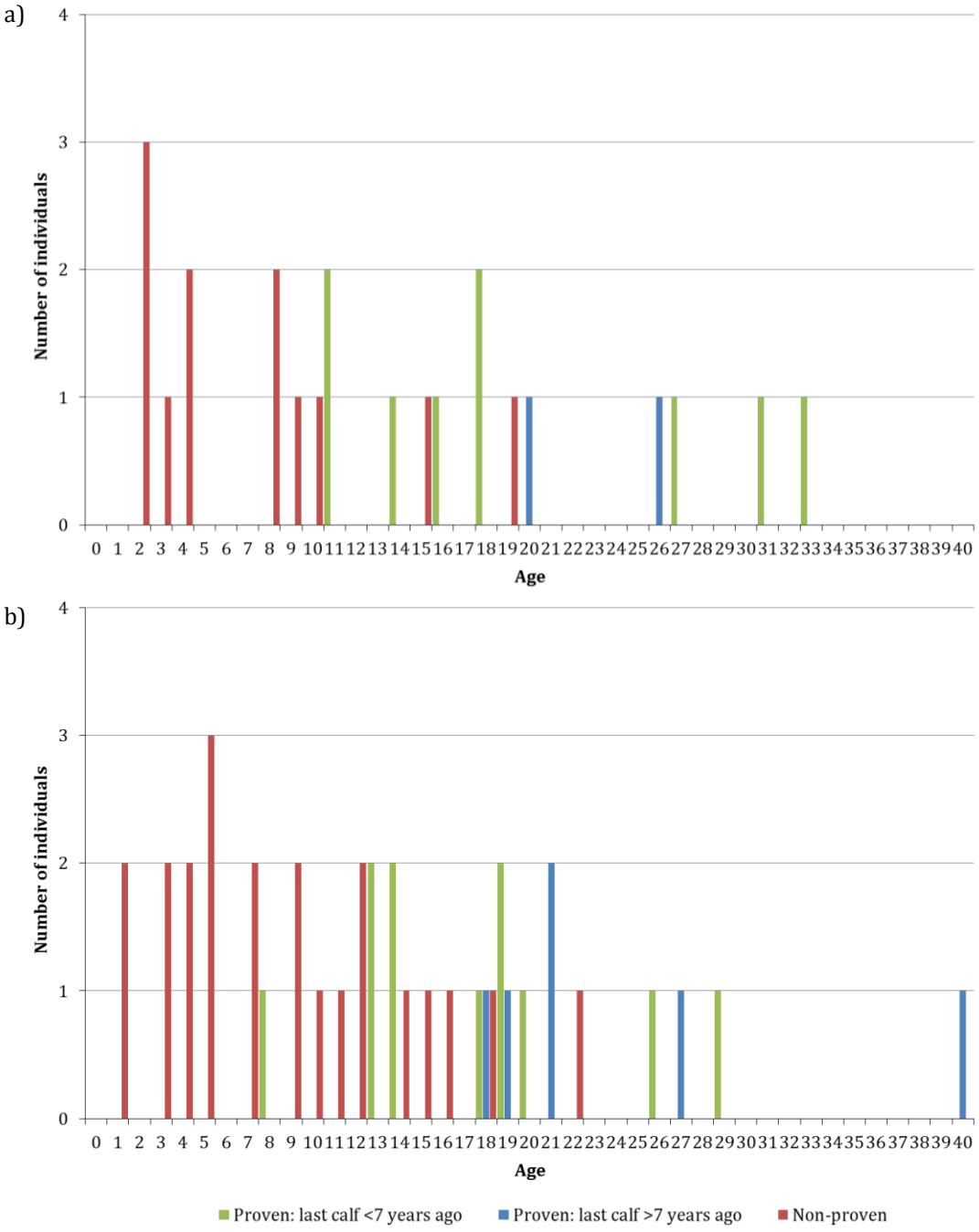


Figure 5.1: Age and breeding status of a) male and b) female black rhinoceros in the European population at the end of 2010, and included in the study.

5.2.2. Faecal sample collection and preparation

A total of 11,222 faecal samples (9743 female samples and 1479 male samples) were collected over a sample collection period that ranged between 4 months, and 6 years; the latter as part of a routine reproductive monitoring programme for females at Chester Zoo. Faecal samples were collected at least weekly from males, and every other day from females across the monitoring period. In 27 individuals (9 males and 18 females), samples were collected for over 12 months, to rule-out any seasonal differences in hormone metabolite concentration or cyclicity patterns. Samples were collected by keepers as soon as possible after defecation, taking multiple sub-sections from different areas of the faecal bolus to allow for potential uneven distribution within the sample, and combined in a zip-lock plastic bag. Samples were then frozen at -20°C following collection, and stored before shipment to Chester Zoo, UK for analysis.

Hormone metabolites were extracted from faecal samples according to an established wet-weight shaking extraction method (Edwards et al. 2013; Walker et al. 2002) (see Chapter 2 section 2.2.3 and Appendix 1 for detailed description of methods and protocols respectively). In brief, each sample was thawed, thoroughly mixed and weighed (0.5g ± 0.003g), before adding 5ml 90% methanol, vortexing and shaking overnight on an orbital shaker. Each sample was then vortexed and centrifuged for 20 minutes at 598g. The supernatant was decanted, dried under air, re-suspended in 1ml 100% methanol and the resulting faecal extract stored at -20°C until analysis.

5.2.3. Enzyme immunoassay

Previously described enzyme immunoassays adapted from Munro and Stabenfeldt, (1984), were used with some modifications to measure faecal progesterone (Walker et al. 2008) and testosterone (Edwards et al. *in prep*) metabolites (see Chapter 2 section 2.2.4 and Appendix 1 for detailed description of methods and protocols respectively). Each EIA utilised an antiserum (monoclonal progesterone CL425 or polyclonal testosterone R156/7; C.J. Munro, University of California, Davis); corresponding horseradish peroxidase (HRP) conjugated label (C.J. Munro, University of California, Davis); and standards (Sigma-Aldrich, UK) on a Nunc-Immuno Maxisorp (Thermo-Fisher Scientific, UK) microtitre plate. Black rhino faecal extracts were diluted as

necessary in EIA buffer (female 1:70 for progesterone (or 1:1050 during pregnancy) and males 1:20 for testosterone), and run in duplicate (50µl) on the respective EIA's.

5.2.4. Biochemical validation

EIAs were biochemically validated for measuring 1) progesterone metabolites in female and 2) testosterone metabolites in male black rhino faecal extracts through parallelism 1) R_2=0.969, $F_{1,7}$=222.140, P<0.001; 2) R_2=0.997, $F_{1,7}$=2563.486. P<0.001) and matrix interference assessment 1) R_2=0.998, $F_{1,7}$=4338.484, P<0.001; 2) R_2=0.996, $F_{1,7}$=1668.608, P<0.001) (see Chapter 2, section 2.4 for full details). Intra- and inter-assay CVs for progesterone and testosterone EIAs were 17.3%, 14.6% and 12.9%, and 12.9%, 7.2% and 8.5% for high and low binding synthetic and biological controls respectively. The cross reactivities for testosterone and progesterone antisera have been reported elsewhere (deCatanzaro et al. 2003; Walker et al. 2008) (See Appendix 2 for full details).

5.2.5. Oestrous behaviours

Concurrent with the faecal sample collection, keepers that work with the rhinos on a daily basis were asked to complete a number of questions regarding oestrous behaviours observed from the study females. In particular, keepers were asked to select from a list of potential behaviours (listed in Table 5.2), which were regularly expressed during oestrus by a given female, and the regularity (monthly, irregular, not seen) with which that female was considered to be in oestrus during the study period.

Table 5.2: Potential oestrous behaviours described in female black rhinoceros.

Female oestrous behaviours
+/- Restlessness/pacing [4]
+/- Vocalisations [3]
+/- Increased urine spraying [1,2,3,4]
+/- Tail up [5]
+/- Swelling of vulva [2,3]
+/- Vulva wink [4]
+/- Discharge [2]
+/- Less cooperative with keepers (or) more cooperative with keepers
+ Interest in male
+ Female presenting hind-quarters to male [2]
+ Female standing for male [2,3]

+/- may be seen with or without male present; + only seen when male present

[1] Hutchins and Kreger (2006)

[2] Goeltenboth et al. (1995)

[3] Fouraker and Wagener (1996)

[4] Berkeley et al. (1997)

[5] Hitchins and Anderson (1983)

5.2.6. Body condition

Body condition scoring (BCS) involves the visual assessment of specific parts of the body for muscle and fat content, and can be a useful indicator of general health condition of an individual. A standardised body condition scoring system as previously developed for *in situ* black rhinoceros (Reuter and Adcock 1998), which assesses seven key areas of the body on a five-point scale, was applied to the *ex situ* population. Body condition assessment was first conducted at four institutions, using a combination of direct assessment and photographs, to score individuals according to the standardised protocol. Once these two methods of assessment were consistent, BCS was done for all other institutions included in the study using photographs alone. Photos of each rhino were taken from multiple angles; a front view of the rhino, ideally with its head up, to assess condition of the neck region; a side-view, preferably while standing still; and a rear view, when the rhino was standing with its tail down. All photos were taken avoiding bright sunlight which can prevent accurate assessment of body condition. Overall body condition scores were assigned to each individual, using 0.5 point increments between 1 and 5, with a BCS of 1.0 being considered to be very poor/emaciated, 2.0 as poor/thin, 3.0 as average/fair, 4.0 as good/ideal, and 5.0 as excellent/heavy (Reuter and Adcock 1998).

5.2.7. Data analysis

In females, samples collected at least every other day were analysed for progesterone metabolite concentration and used to investigate reproductive cyclicity. Oestrous cycles were determined from faecal progesterone metabolite concentration (PG), and characterised according to a previously established method, where samples with baseline hormone concentrations are distinguished from those with elevated hormone concentrations, using an iterative process (Brown et al. 2001; Brown et al. 1994b). All non-pregnant samples from an individual female were used to calculate the mean and standard deviation (SD). An iterative process was then used to remove all samples greater than 1.5SD above the mean, before the mean was re-calculated and the process repeated until no samples exceeding 1.5SD from the mean remained. These samples were considered to have baseline concentrations of PG, and represented the follicular phase of the cycle. The onset of the luteal phase was considered to be the first sample where PG concentration exceeded 1.5SD above the mean, and the end of the luteal

phase was considered to be when at least two consecutive samples were below the threshold of 1.5SD of the mean. This cyclic pattern represents one oestrous cycle. Cycle length was calculated from the last sample of the follicular phase prior to the increase in PG concentration, to the same point on the following cycle. As per previously established criteria (Brown et al. 2001), sustained periods (> 10 days) where PG concentration remained at baseline without any increase above this threshold of 1.5 SD above the mean were categorised as acyclic periods.

To investigate differences in testosterone metabolite concentration between males, weekly samples were analysed for hormone metabolite concentration, and compared using generalised linear mixed models (GLMM's) in MLwiN version 2.02 (Rasbash et al. 2005). GLMMs allow nested random effects to be incorporated into the model (Bolker et al. 2009) to control for relatedness of data, such as repeated faecal samples per subject. Normality tests were conducted in IBM® SPSS® statistics version 20, and hormone data were transformed where necessary, using $\log_{10}$ transformations to improve the distribution of data. To investigate the effect of explanatory variables on hormone concentration, random (date of sample collection and subject ID) and fixed effects, either categorical (age class, reproductive category) or continuous (age) were incorporated into a GLMM. Within categorical GLMM's, a reference category was assigned, to which all other categories were compared (reproductive class age 17-33 and proven breeders respectively). A normal error structure was used for all models of $\log_{10}$hormone metabolite concentration, and the significance of each fixed effect was determined using the Wald statistic and chi-squared (χ^2) distribution, with alpha set to 0.05.

Differences in the relative proportions of different cycle types observed between proven and non-proven females were investigated using cross-tabulation with Pearson's chi-square test, in IBM® SPSS® statistics version 20. The percentage of each cycle type across reproductive categories was compared using z-tests, with P-values adjusted with the Bonferroni method. To investigate any potential differences in body condition (males and females) and the regularity of oestrus (females only) across reproductive categories, comparisons were made using either Mann Whitney U tests (two groups) or Kruskal Wallis (three or more groups), both conducted in IBM® SPSS® statistics version 20.

5.3. Results

5.3.1. Reproductive cyclicity and breeding status in female black rhinoceros

Oestrous cycles, as determined by faecal progesterone metabolite concentration (section 5.2.7), were observed in all but two of reproductive-age females (age 5-32; 29/31). However, of the two females where no evidence of cyclicity was observed, one female was pregnant throughout the monitoring period, and another had given birth but had not yet resumed cyclicity post-partum. Therefore, with these two females excluded, 100% of mature females exhibited at least some cyclicity during the study period. In the immature age class (n=7 females), young females age 4y 11m, 4y 4m, 4y 3m and 3y 8m were already showing clear signs of cyclicity, whereas one female age 4y 3m, and two females aged less than 3 years were not yet showing signs of cyclicity. This indicates that although females are considered mature at age five, they may commence reproductive cyclicity earlier than this, but may also show individual variation. Additionally, one female considered to be post-reproductive still showed signs of cyclicity at age 40.

A total of 436 oestrous cycles were characterised during this study (N=34 females age range 3y 8m-40y); with cycles observed during all months of the year, indicating no seasonal differences in cycle occurrence. However, there was substantial variation in cycle lengths both within and between females (Figure 5.2). The majority of cycles ranged from 20-40 days (63.3%); with an average length of 27.1days (standard deviation 5.0 days). However, irregular cyclicity was also observed, including short (< 20 days; 12.4%) or long (> 40 days; 14.7%) cycles, and prolonged periods where faecal progesterone metabolite concentration remained at baseline (hereafter termed acyclic periods), ranging from 12-127 days (9.6%) (Figure 5.3).

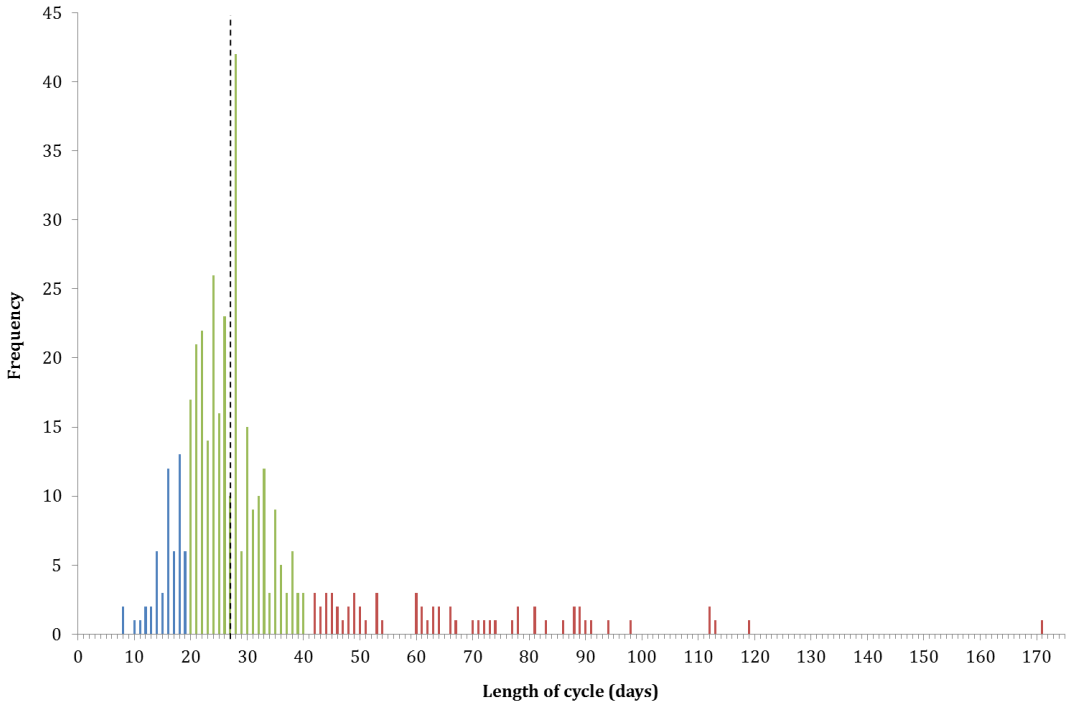
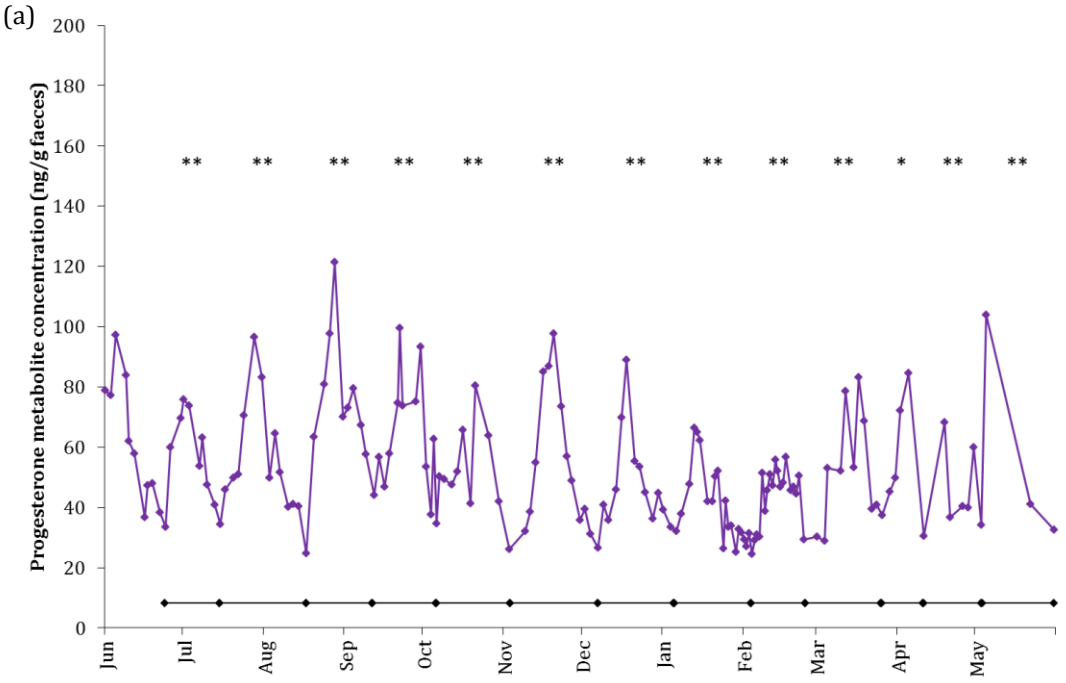


Figure 5.2: Distribution of oestrous cycle lengths as determined from faecal progesterone metabolite concentration, according to iteration method (Brown et al. 2001; Brown et al. 1994b). Cycles were categorised as <20 days (blue), 20-40 days (green) or >40 days (red) in length. In accordance with other studies, cycles of 20-40 days in length were considered normal (Roth 2006), with mean (±SD) length 27.1 (±5) days (dashed line).

(a)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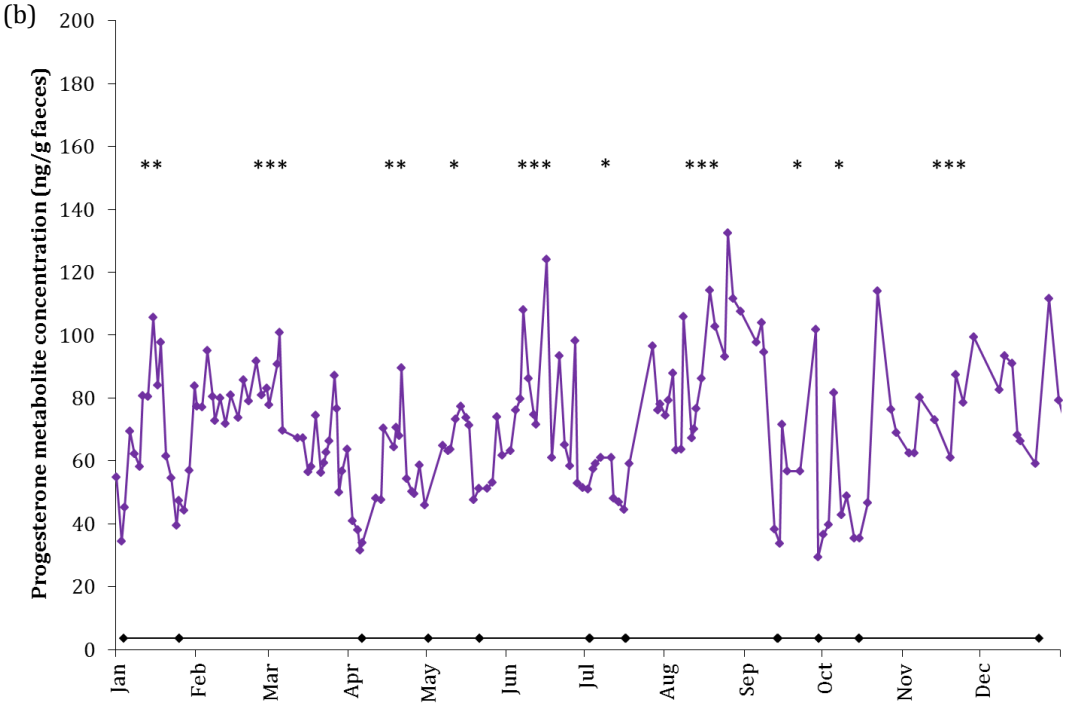
(b)


Progesterone metabolite concentration (ng/g faeces)
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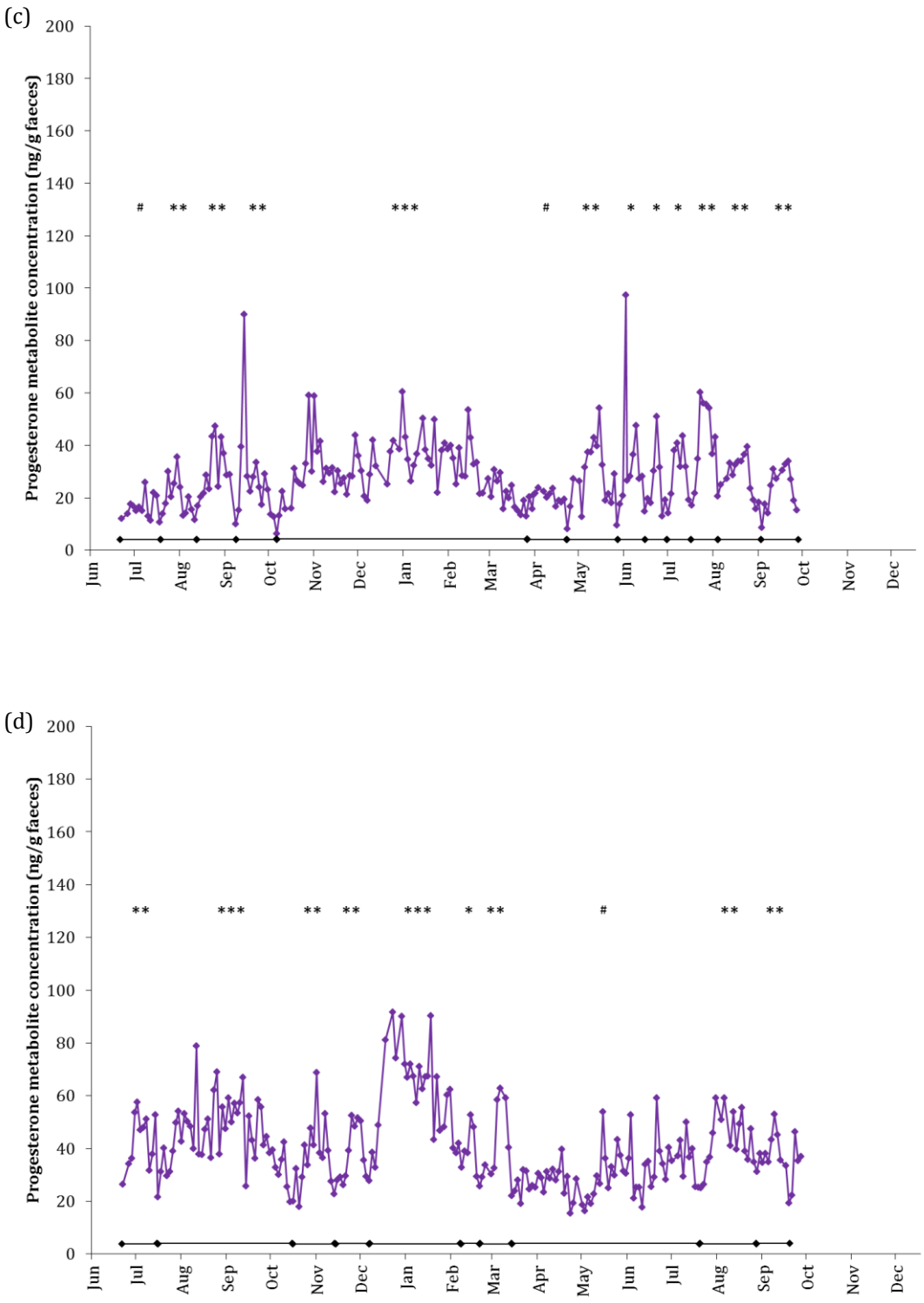

Figure 5.3: Four individual profiles of faecal progesterone metabolite concentration illustrating periods of regular cyclicity, with cycles 20-40 days in length (**), and periods of irregular cyclicity, including extended luteal phases characteristic of long cycles >40 days in length (***), short cycles <20 days in length (*), and acyclic periods (#).

A total of 411 oestrous cycles were characterised within females of reproductive age (5-32) during the study period, 263 were categorised as regular cycles of 20-40 days in length, whereas 148 were categorised as irregular cycles (<20 days, >40 days and acyclic periods combined) (Table 5.3). The occurrence of these regular and irregular cycles were compared by reproductive category, but there was no difference between the percentage of cycles classed as regular and the percentage classed as irregular between proven females (N=14) and non-proven females (N=15) (χ^2=0.548, df=1, P=0.459).

However, when comparing the occurrence of the four different cycle types (<20 days. 20-40 days, > 40 days and acyclic periods) across reproductive categories, the relative proportion of cycle types observed were different between proven and non-proven females (χ^2=9.116, df=3, P=0.028). Further tests revealed that there was no difference in the proportion of<20d cycles, 20-40d cycles or acyclic periods according to reproductive category (P>0.05), but a higher percentage of longer cycles (>40 days in length) were observed in non-proven females (P<0.05). Furthermore, when comparing these four cycle types between proven females that had produced a calf during the last seven years (N=9), and proven females that had not produced a calf during the last seven years (N=5), again there was a difference in the proportion of different cycle types observed (N=140 cycles) (χ^2=15.282, df=3, P=0.002), with acyclic periods more common in females that had not reproduced during the last seven years (P<0.05), but no difference in the proportion of other cycles types (P>0.05).

These differences in cycle type occurrence between females of different reproductive categories are further exaggerated if we consider not only the frequency of cycles observed, but also the proportion of the study period that females exhibited the different cycle types (Table 5.4, Figure 5.4). Each cycle was characterised as described above, and the total number of days that a particular female was exhibiting short cycles< 20 days in length were added together. This was repeated for 20-40 day cycles, > 40 day cycles and acyclic periods to give the proportion of the study period days during which each cycle type was observed. As many of the long cycles (>40 days) that occurred during the course of this study were at least two or three times the length of a normal cycle in this species (here categorised as 20-40 days) (Figure 5.2), only 49% of sampling days in non-proven females were considered to be normal periods of cyclicity compared to 63% for proven females.

Table 5.3: Number (and percentage) of cycles of each type exhibited by females in each reproductive category during the study period.

	Non-Proven	Proven	Proven and has bred during the last 7 years	Proven but has not bred during the last 7 years
<20	28 (10%)	21 (15%)	15 (14%)	6 (19%)
20-40	170 (63%)	92 (67%)	75 (71%)	17 (53%)
>40	52 (19%)*	12 (9%)	11 (10%)	1 (3%)
acyclic	21 (8%)	13 (9%)	5 (5%)	8 (25%)*
Total cycles	271	138	106	32
Number females	15	14	9	5

* Denotes differences significant at P<0.05 level

Table 5.4: Total number of days (and percentage) that females in each reproductive category exhibited each cycle type during the study period.

	Non-Proven	Proven	Proven and has bred during the last 7 years	Proven but has not bred during the last 7 years
<20	449 (5%)	333 (9%)	241 (8%)	92 (12%)
20-40	4638 (49%)	2449 (63%)	2031 (65%)	418 (54%)
>40	3583 (37%)	737 (19%)	695 (22%)	42 (5%)
acyclic	892 (9%)	358 (9%)	143 (5%)	215 (28%)
Total days	9562	3877	3110	767
Number females	15	14	9	5

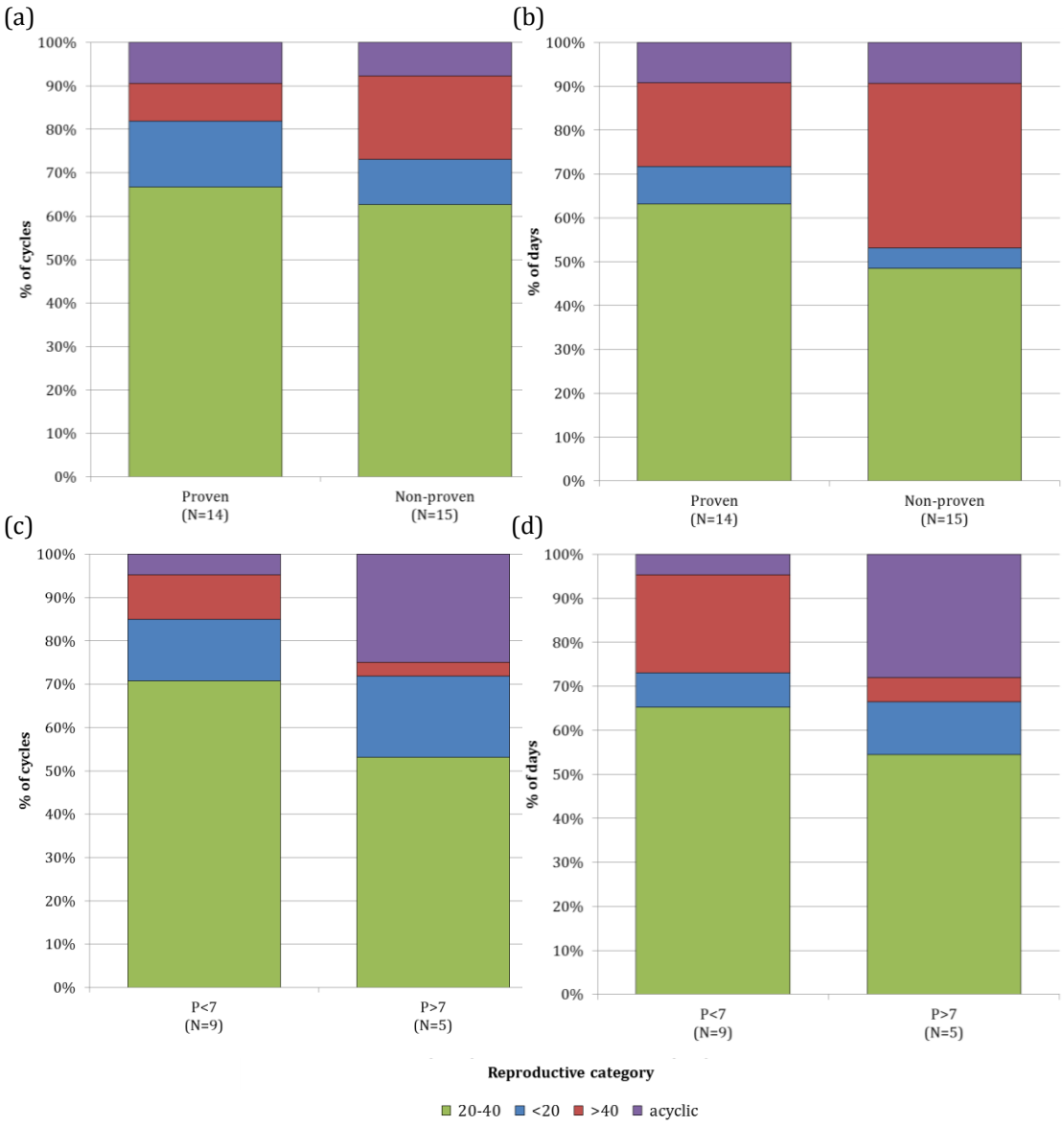

Figure 5.4: Percentage of oestrous cycles (a and c) and study period days (b and d) when females within different reproductive categories were characterised as exhibiting cycles of <20 days, 20-40 days, >40 days, or acyclic periods; (a and b) proven breeders compared to non-proven breeders, and (c and d) proven females that had produced a calf within the last 7 years compared to those that had not.

5.3.2. Oestrus behaviours

The frequency with which keepers reported potential oestrus behaviours to be present or absent in the study females are presented in Figure 5.5. Of these, increased urine spraying was the most commonly reported oestrus behaviour seen by keepers (N=22 females), and was observed even if a male was not present. Presenting hind-quarters to the male (N=21), standing for the male (N=21) and showing interest in the male (N=16) were also commonly reported when a male had been present.

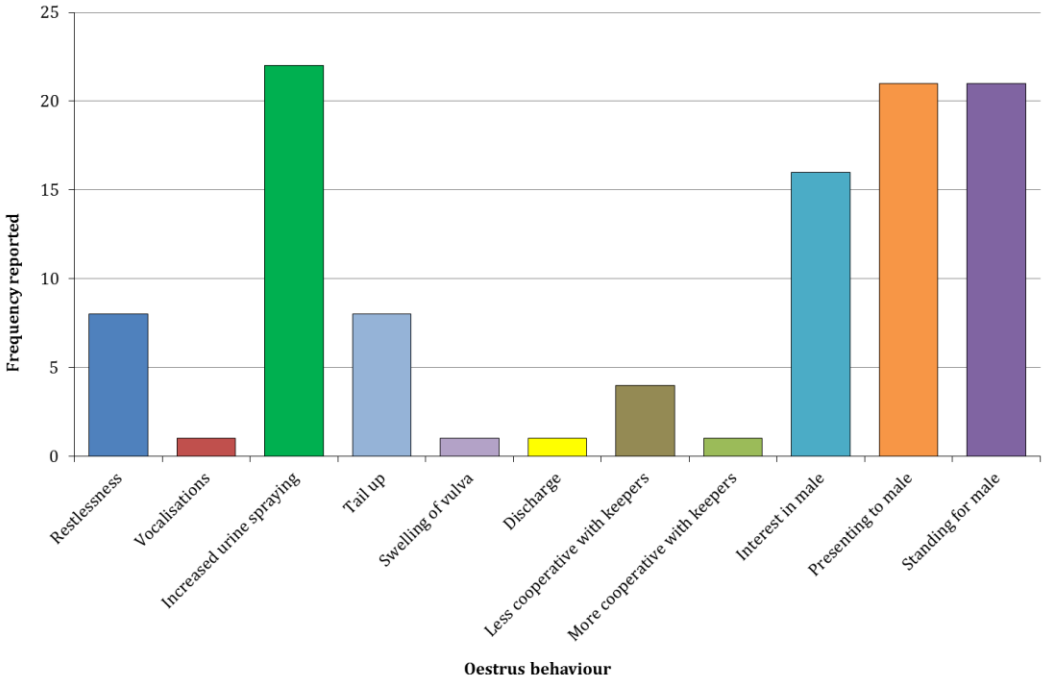

Figure 5.5: Behaviours that were observed from females when in oestrus and the number of females for which that particular behaviour was reported.

However, among reproductive-age females (N=31), the relative regularity of expression of oestrus (rated as being monthly, irregular or not seen) varied between females according to reproductive category. Oestrus was observed monthly more often in proven females than non-proven females (Mann Whitney U=56.000, P=0.006). Furthermore, when comparing the three reproductive categories of proven and had bred during the last seven years, proven but had not bred during the last seven years, and non-proven, there was also an overall difference in regularity of observed oestrus (Kruskal Wallis =10.299, df=2, P=0.006; Figure 5.5). Between category tests confirmed that females that had bred during the last 7 years showed oestrus more regularly than

non-proven females (Mann Whitney U=9.812, P=0.007), however, there was no difference between proven females depending on whether they had reproduced during the last seven years (Mann Whitney U=-1.845, P=1.000), or between females that had not bred during the last seven years and those that had never bred (Mann Whitney U=7.967, P=0.168).

Oestrus behaviours were reported as being observed monthly in 73% of females that had reproduced within the last 7 years, compared to 60% of females that have bred previously but not produced a calf in the last 7 years, and only 13% of non-proven females. Among non-proven females, a further 13% were recorded as never exhibiting clear behavioural signs of oestrus, and 73% only exhibit oestrous behaviours irregularly (Figure 5.6).

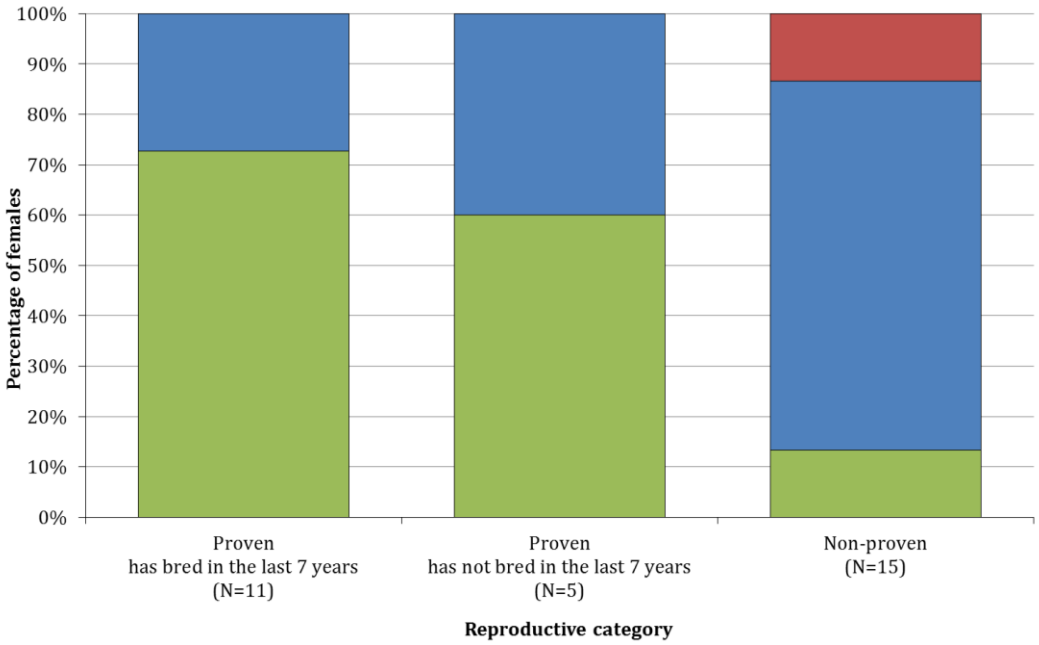

Figure 5.6: Percentage of females that keepers reported as being in oestrus monthly (green), irregularly observed (blue) or not seen in oestrus (red) in proven females that have produced a calf during the last 7 years, proven females that have not produced a calf during the last 7 years, and non-proven females.

5.3.3. Testosterone and breeding status in male black rhinoceros

Using all faecal samples collected across the study period, age was a significant predictor of faecal testosterone metabolite concentration with $\log_{10}$ Tt increasing with age (GLMM χ^2=17.747, df=1, P<0.001) (Figure 5.7), and immature males in age class 1-7 exhibiting significantly lower $\log_{10}$ Tt than males age 7-17 (GLMM χ^2=8.715, df=1, P=0.003), 17-33 (GLMM χ^2=24.478, df=1, P<0.001) and 33+ (GLMM χ^2=4.270, df=1, P=0.04), and males age 7-17 lower $\log_{10}$ Tt than 17-33 (GLMM χ^2=5.700, df=1, P=0.017).

Controlling for age (age class as an additional random effect), males that had produced a calf in the last 7 years had higher $\log_{10}$ Tt than non-proven males (GLMM χ^2=7.730, df=1, P=0.005). Proven males that had not bred during the last seven years tended to exhibit intermediate $\log_{10}$ Tt concentration relative to those that have never sired a calf and those that have bred more recently (proven and bred within seven years vs. proven but not bred within seven years GLMM χ^2=0.197 P>0.05; proven but not bred within seven years vs. non-proven GLMM χ^2=1.955 P>0.05) (Figure 5. 8). Overall, non-proven males had significantly lower $\log_{10}$ Tt than proven males, regardless of whether they had bred during the last seven years or not (GLMM χ^2=7.599, df=1, P=0.006).

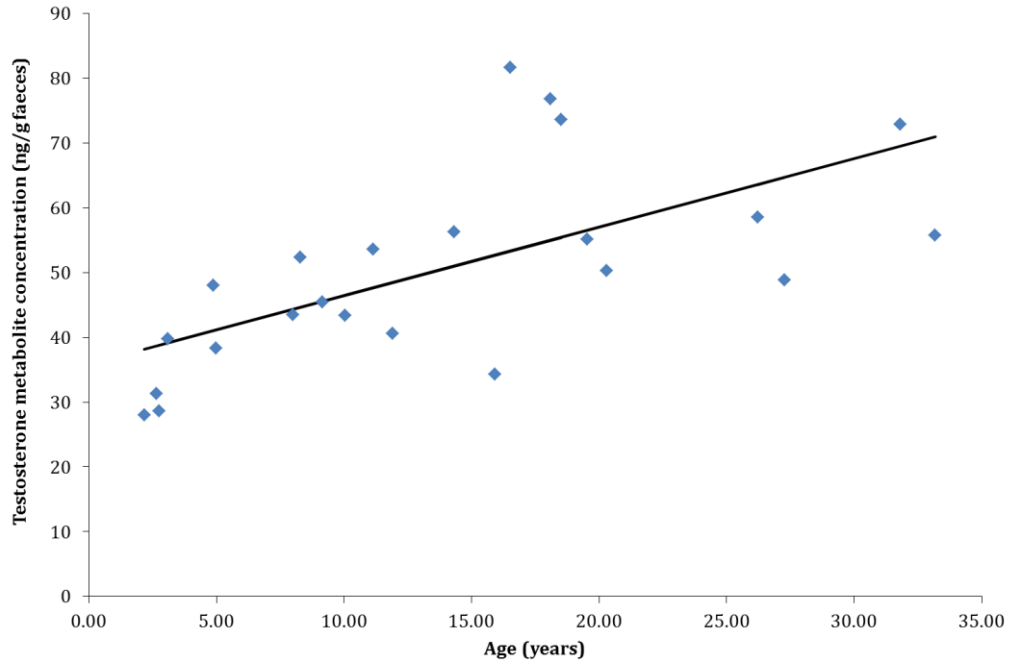

Figure 5.7: Relationship between age and average faecal testosterone metabolite concentration in male black rhinoceros. Figure represents the prediction obtained from GLMM model which includes all samples collected over the study period (line) overlaid onto the average faecal testosterone metabolite concentration calculated for each male.

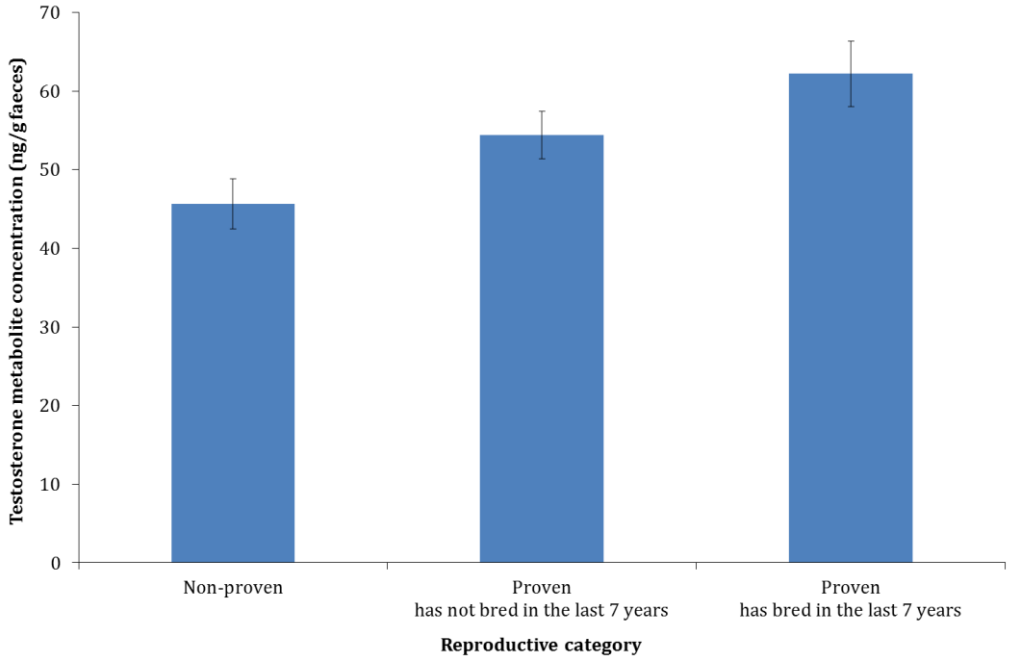

Figure 5.8: Faecal testosterone metabolite concentration (±s.e.m) in males that have never bred, compared to those that have bred but not in the last 7 years, and those that have bred in the last 7 years.

5.3.4. Body condition and reproductive success

Among all females (N=39), body condition scores (BCS) ranged from 3.0-4.5. There was no difference in BCS according to age (Mann Whitney U=92.500, P=0.157). However, non-proven females had higher BCS than proven females (Mann Whitney U=52.500, P=0.004), with 9 of 15 non-proven females scored as 4.5, compared to only 1 of 17 proven females (Figure 5.9). When the reproductive-age females (5-32, N=31) were categorised according to whether they had bred during the last seven years, again non-proven females scored higher than proven females that had bred during the last seven years (Kruskal Wallis =9.000, P=0.009), but there was no difference between those females that had not bred during the last seven years to either those that had (Kruskal Wallis =-0.900, P=1.000) or those that had never bred (Kruskal Wallis =8.100, P=0.117).

However, in males, BCS was more consistent across individuals, in fact only a single male scored 3.5, with the remaining males all scored as 4.0. There was no difference in BCS according to age (Mann Whitney U=45.000, P=0.858), or according to reproductive categories (proven vs. non-proven (Mann Whitney U=31.500, P=0.740)).

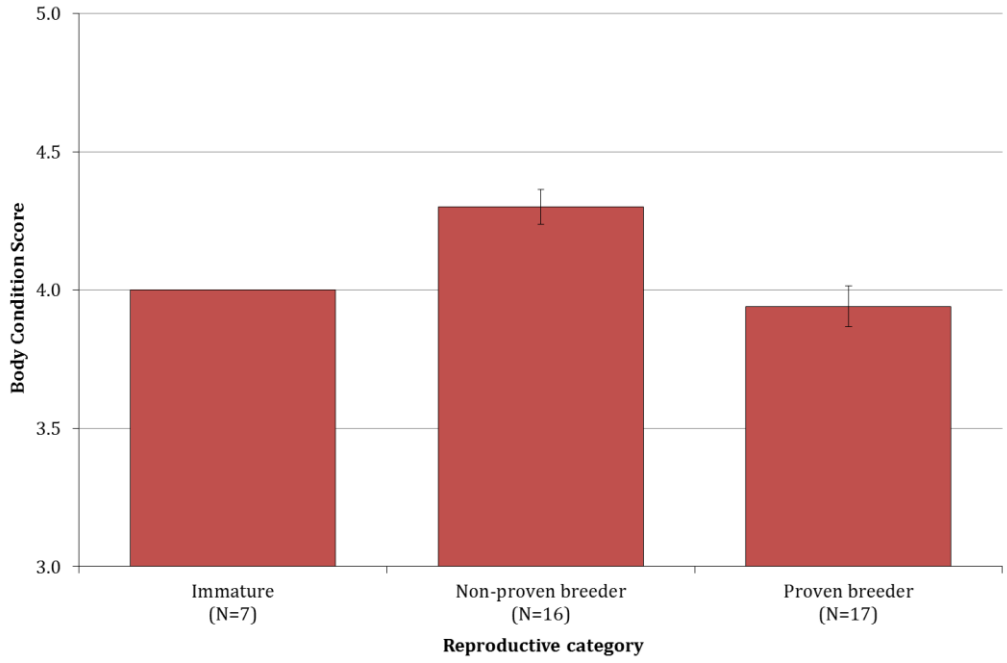

Figure 5.9: Mean body condition score (BCS) (±s.e.m) across females, according to reproductive category. A BCS of 1.0 is considered to be very poor/emaciated, 2.0 as poor/thin, 3.0 as average/fair, 4.0 as good/ideal, and 5.0 as excellent/heavy (Reuter and Adcock 1998).

5.4. Discussion

5.4.1. Female black rhinos and reproductive success

The average cycle length of females in the European captive population estimated from this current study of 27.1±5 days is comparable to that from endocrine data published from other studies, both *in situ* (26.8±1day; (Garnier et al. 2002)) and *ex situ* (21-22 days (Hindle et al. 1992); 26±1.4 days (Radcliffe et al. 2001); 26 days (Berkeley et al. 1997); 24 and 26.5 (Schwarzenberger et al. 1993); 26.8±0.5 days (Brown et al. 2001)). Similar to the survey of captive black rhinoceros in America by Brown et al. (2001), cyclicity in the EEP population was quite erratic, with cycles both longer and shorter than average also seen, and periods of acyclicity. Interestingly, although periods of regular and irregular of cyclicity were observed in both proven and non-proven females, the longer cycle type (>40 days in length) were exhibited more often in non-proven females. Furthermore, among proven females, those that had not reproduced during the last seven years exhibited a higher proportion of acyclicity than those that had bred more recently.

In white rhinoceros females, the phenomenon of two cycle types (long vs. short) was identified some time ago (Brown et al. 2001; Patton et al. 1999; Schwarzenberger et al. 1998), and generally the shorter cycle length (30-35days) is considered to be fertile (Patton et al. 1999; Radcliffe et al. 1997; Schwarzenberger et al. 1998), whereas the longer cycle (65-70days) is considered to be abnormal since no conceptions have been known to occur during these cycles (Brown et al. 2001; Roth 2006; Schwarzenberger et al. 1998). Although we cannot yet state that these longer cycles observed in the black rhino are abnormal, we have not seen any conceptions occur during these periods of irregular cyclicity (Edwards et al., *unpublished data*), suggesting they may not be a fertile cycle type. At the very least, these longer cycles (and acyclic periods) may last 2-3 times the length of an average cycle, which reduces the opportunities for mating and conception. If these cycles are considered to be abnormal, this has important consequences for breeding management, as non-proven females exhibited regular periods of cyclicity for only 49% of the study period.

However, the etiology of these different cycle lengths in African rhinos is still not clearly understood, and the question remains as to whether they are indeed indicative of a problem. As yet, insufficient long-term monitoring of reproductive cyclicity has been conducted in either species *in situ*, to determine whether this is a normal occurrence in wild rhinos, or whether it is specific to captivity. However, in wild black

rhinos, Hitchins and Anderson (1983) reported inter-oestrus intervals ranging from 26-46 days, and Garnier et al. (2002) reported three-quarters of cycles had a mean length (±s.e.m) of 26.8±1day, but the remainder of cycles were characterised by either an extended luteal phase or extended follicular phase, indicating that these irregular periods of cyclicity may not be confined to captive black rhinos.

One possibility is that this phenomenon may be due to seasonality (Garnier et al. 2002), as is the case in mares, where luteolytic failure and extended luteal phases are seen during the transition between breeding and non-breeding seasons (King et al. 2010). This may be a possibility in wild populations where births are often more seasonal according to rainfall (Garnier et al. 2002), and has been proposed as an explanation for the presence of anovulatory follicles observed with ultrasound (Radcliffe et al. 2001). However, within this captive population, both longer cycles of >40 days and the more typical 20-40 day cycles were observed across all months of the year, similar to that reported in the American population of black rhinos (Brown et al. 2001), and for white rhinos (Brown et al. 2001; Patton et al. 1999; Schwarzenberger et al. 1998), suggesting that this may not be just a seasonal occurrence.

Another possibility for the occurrence of these different cycle types is that they may be related to age; or more specifically to asymmetric reproductive aging (Hermes et al. 2004). In both black and white rhinoceros females, long periods without reproduction can have detrimental effects on their reproductive system, leading to the development of pathologies, reduced fertility, and irreversible acyclicity leading to premature senescence. It is thought that prolonged exposure to endogenous sex steroids during continuous cyclicity without conception play a role in this decline, as females that fail to reproduce by the age of 16 may have already exhibited as many oestrus cycles as a regularly breeding female would in her entire lifetime. This phenomenon is also seen in captive elephants, where a non-breeding female's active reproductive lifespan may be shortened by as much as 15 years compared to that of a breeding female (Hildebrandt et al. 2000). The constant exposure to ovarian sex steroids have been linked to the development of reproductive pathologies, which together with the exhaustion of finite numbers of follicles, reduced chances of conception due to oocyte viability and uterine function, and reduced capability of corpora lutea to support early pregnancy, the chances of reproduction also decline with age (Hermes et al. 2004).

However, in the EEP population irregular cycles were observed in females between the ages of 5-30 years, indicating that age alone does not explain all of the reproductive

issues in this population. Nonetheless, with eight out of the 22 non-proven females already over the age of 15, and a further 10 between the ages of 10-15, the factors limiting reproduction in this population need to be investigated before these females are no longer able to reproduce. However, there is a further caveat with age that should be considered, as older individuals may be more likely to be proven breeders than younger individuals, due to the fact that they have had a longer time period where they could have reproduced. Although the analyses used here only considered males over the age of 7 years and females over the age of 5 years when comparing proven and non-proven breeders, the possibility remains that those younger non-proven individuals may not have had sufficient opportunity to reproduce to be directly comparable to the older non-breeding animals. More detailed analyses would be beneficial to investigate whether the intrinsic differences observed between proven and non-proven breeders could in part be due to endocrine correlates of age.

There is as yet no clear pattern in when or why these cycle types are observed, but there do appear to be a higher proportion of longer cycles among non-proven females, which may in part explain some of the variation in reproductive success. However, further investigation is necessary to understand the physiology and potential pathology relating to these different cycle types that seem to occur in both black and white rhinoceros populations. In particular, investigating what may be occurring within individual females, at the time that these different cycles occur, would be beneficial in understanding their potential causes.

One factor that may be related to reproductive success as indicated by this study is that of body condition score. Non-proven females were scored as having higher body condition than proven females, which could be one factor involved in lower reproductive success. Females that are classed as overweight or obese may have reduced oocyte quality and may be at increased risk of failed implantation and pregnancy loss (Brewer and Balen 2010). However, what may be of more relevance to this study is the potential disruption to ovarian cyclicity. In a study by Vick et al. (2006), obese mares exhibited longer cycles and had longer luteal phases than food restricted mares. The extended luteal phase may have indicated a persistent corpus luteum, or the luteinisation of an anovulatory follicle (McCue and Squires 2002). The persistence of anovulatory follicles, has been reported in a number of species (Lopez-Gatius et al. 2001; Veiga-Lopez et al. 2006; Wiltbank et al. 2002), and their presence during extended cycles may suggest that the mechanism of ovulation may be

suppressed in obese females. As longer cycles were more common in non-proven females, who were also scored higher on body condition scores, this is one potential explanation for reduced reproductive success among females.

As well as the incidence of irregular cyclicity in this population, a further issue that has been highlighted is the difference between breeding and non-breeding females in how regularly oestrous behaviours were observed. Non-proven females display overt signs of oestrus less often than proven females, with some females failing to demonstrate clear behavioural signs of receptivity despite faecal progesterone metabolite profiles indicating that they should have been in oestrus. This absence of overt oestrous behaviour has been reported in a wide range of other species including cattle (Allrich 1994), tapir (Brown et al. 1994a), giant panda (Kersey et al. 2010), and Arabian leopards (van Dorsser et al. 2007), and is a commonly reported issue in this population (M. Pilgrim *pers. comm.*). This lack of oestrous behaviour could have potential management implications as if females do not express suitable behaviours, introduction to a potential mate may not be made. Alternatively, if introductions do occur but the female is not receptive to the male, aggression can ensue. This could potentially result in introductions being made less often, and may be less successful when they are made, leading to a lower chance of conception. It is therefore important to understand why non-proven females are not expressing behavioural signs of oestrus as regularly as proven females.

The expression of oestrous behaviours is dependent on the correct balance of hormones prior to ovulation. Oestradiol is produced by the maturing follicle, which in the relative absence of progesterone prior to ovulation, eventually reaches a threshold after which oestrous behaviours are expressed and the female becomes receptive to the male (Allrich 1994). However, if oestradiol concentration is insufficient (Bennett et al. 1991), for example because progesterone is too high (Asa et al. 1984), or inhibited by glucocorticoid secretion (Asa and Ginther 1982; Stoebel and Moberg 1982), oestrus behaviours may not be expressed. This has so far received little attention in the black rhinoceros, but constitutes a significant management concern, due to the serious aggression that can ensue between black rhino pairs (Fouraker and Wagener 1996), particularly if the female is not fully receptive. As a difference in behavioural expression has been observed between proven and non-proven females, there is therefore a need to investigate whether there may also be differences in oestradiol

concentration either between females, or between periods of regular and irregular cyclicity, which may contribute to reduced reproductive success (see Chapter 7).

5.4.2. Male black rhinos and reproductive success

As expected, male testosterone concentration increased with age (Asa 1996; Christensen et al. 2009; Kretzschmar et al. 2004), with immature males exhibiting significantly lower faecal testosterone metabolite concentrations than mature males. Interestingly, there was also clear difference between proven and non-proven males, with the latter exhibiting significantly lower testosterone concentrations. This indicates that there may be a relationship between testosterone concentration and reproductive success in male black rhinoceros, which could be one possible explanation for the reproductive skew observed within this population. However, what we cannot yet determine is whether proven males have higher testosterone due to their breeding status, or whether underlying differences between males may result in both higher testosterone and higher reproductive success.

Unlike the relationship observed in females in this study, there was no relationship between body condition score and reproductive success. In fact, all but one of the males included here were scored the same, indicating that body condition appears to be more consistent among males. Although body condition can be associated with differences in testosterone concentration and sperm quality (Michalakis et al. 2013), this does not seem to explain the differences in reproductive success observed within this population.

In polygynous species such as the black rhinoceros (Garnier et al. 2001; Hutchins and Kreger 2006), males will compete for access to receptive females, often with the dominant male monopolising most of the matings (Garnier et al. 2001). Increased testosterone concentration is associated with dominance in a range of species (Beehner et al. 2006; Li et al. 2004; Mooring et al. 2004; Negro et al. 2010; Rachlow et al. 1998), and plays an important role in male:male competition and aggressive encounters (Gleason et al. 2009). However, in captive populations, mature male black rhinos are generally kept apart, and may often be the only mature male at an institution, meaning elevated testosterone may not be required for male:male competition. However, testosterone may also mediate breeding behaviour and libido in

male:female sexual encounters (Deen 2008; Gleason et al. 2009; Roser 2008), and as such, low concentration in non-breeding males may result in reduced motivation or expression of breeding behaviour needed for successful introductions and mating success.

The role of testosterone in both male:male interactions and male:female interactions are supported by the finding by Christensen et al. (2009), that testosterone concentration in captive back rhino males in America was correlated with the presence of both other males, and females. This indicates that testosterone concentration could be related to extrinsic factors, such as the social environment, and it is therefore important to determine whether external factors may also correlate with testosterone and reproductive success in this population (Chapter 6).

It is important to consider that the categorisation of proven and non-proven breeders used in this study does not necessarily take into account whether individuals have had equal opportunities to reproduce. In particular, if a non-proven male and non-proven female have been housed at the same institution, the two individuals' failure to reproduce may not necessarily indicate an issue with both individuals. For example, if a female is not cycling regularly, and not exhibiting overt signs of oestrus, the male may not have had the opportunity to reproduce. Similarly, a female may be cycling normally, and becoming receptive, but the male may not be attempting to mate the female. This is an important factor that needs to be considered when investigating the underlying differences in reproductive success, and when managing populations such as this, where it is not always possible to house sufficient individuals to allow a choice of potential mates.

The aim of this study was to investigate whether any intrinsic differences between breeding and non-breeding males and females were correlated with the observed differences in reproductive success in this population. In summary, non-proven females were more likely to be scored higher on body condition scores, and were more likely to exhibit longer oestrous cycles, potentially coming into oestrus less often. Furthermore, non-proven females were less likely to exhibit overt behavioural signs of oestrous, and as a consequence may be less likely to be introduced to a male for breeding. In males, testosterone concentration was higher in breeding males than non-breeding males, but there were no observed differences in body condition between males of different reproductive status. As the *ex situ* population of black rhinoceros would benefit from both increased reproductive output and reduced reproductive

skew, a better understanding of the factors related to breeding status is essential. Intrinsic factors may play a role in the reduced reproductive success of non-proven male and female black rhinos, but the observed differences in testosterone concentration and occurrence of extended cycles and reduced oestrous behaviour requires further investigation, to understand the underlying physiology of irregular cyclicity in females (Chapter 7), and to determine whether extrinsic factors may help to understand the differences in reproductive success (Chapter 6).

5.5. Conclusion

- Average oestrous cycle lengths observed in this population were similar to those reported elsewhere, with the predominant cycle type between 20-40 days in length, and an average cycle length of 27.1 days.
- However, irregular cyclicity was also common, with shorter (<20 days) and longer (>40 days) cycles, as well as periods of acyclicity also observed.
- Longer cycle types were exhibited more often in non-proven females than proven females, accounting for 19% of all cycles, and 37% of all sampling days.
- Among proven females, periods of acyclicity were more commonly observed in proven females that had not reproduced for more than seven years.
- Non-proven females were also less likely to exhibit oestrous behaviours on a monthly basis than proven females, potentially leading to fewer successful introductions
- Non-proven females also had higher body condition scores, indicating heavier condition than proven females.
- In male black rhinos, non-proven males had lower faecal testosterone metabolite concentration than proven males.
- The potential role of extrinsic factors relating to these observed differences in reproductive success requires further investigation (Chapter 6).
- Further investigation into hormone changes during the oestrous cycle is required, to understand what factors may be associated with irregular cyclicity in females (Chapter 7).

5.6. Acknowledgements

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- Zoo Hannover, Hannover, Germany
- Zoo Krefeld, Krefeld, Germany
- Zoologischer Garten Köln, Köln, Germany
- Paignton Zoo, Paignton, UK
- Port Lympne Wild Animal Park, Hythe, Kent, UK
- Howletts Wild Animal Park, Bekesbourne, Kent, UK
- Zoo Zürich, Zurich, Switzerland
- Zoologischer Garten Magdeburg, Magdeburg, Germany
- Zoo de Pont-Scorff, Pont-Scorff, France

CHAPTER 6:

6. INVESTIGATING THE POTENTIAL INFLUENCE OF EXTRINSIC FACTORS ON ADRENAL ACTIVITY AND REPRODUCTIVE SUCCESS IN THE EEP POPULATION OF EASTERN BLACK RHINOCEROS

Summary

Intrinsic differences in reproductive hormones have been observed between proven and non-proven black rhinoceros in the European captive population (Chapter 5). However, the factors underlying these differences have yet to be determined. As sub-optimal reproduction is currently limiting the growth of this population (Chapter 4), it is important to determine whether aspects of the captive environment may influence reproductive success. One potential area for investigation is the role of adrenal activity, which has previously been illustrated to impact reproduction in a wide range of species, and furthermore has been associated with certain environmental variables, and with mortality in this species.

The aim of this study was to investigate whether social and environmental factors were related to adrenal activity or reproductive success in male and female black rhinoceros (*Diceros bicornis michaeli*) in Europe. Faecal samples from 23 male and 39 female black rhinos were analysed for glucocorticoid metabolites (fGCM), and used alongside questionnaire data collected at 13 institutions across Europe. Social variables included the presence of male and female conspecifics, and their proximity to the subject, either housed with the subject (physical contact) or nearby (visual, auditory and olfactory contact), as well as the total number of males and females at a given institution. Environmental variables included aspects of enclosure design such as the total area, the proportion of solid walls or fencing used for the enclosure boundaries and the percentage access of visitors to within 10m to the individual's enclosure. Finally, keepers were also asked to rate the rhinos in their care on aspects of their behaviour or temperament, to determine how often behaviours such as pacing and charging were observed, how individuals responded to keepers or their environment, and how changeable their behaviour is over time. In both males and females, these variables were analysed with respect to fGCM concentration, and compared between breeding and non-breeding individuals to determine whether extrinsic factors were related to reproductive success. Furthermore, differences in testosterone metabolite (fTt)

concentration in males were also investigated with respect to social and environmental variables.

In females, fGCM concentration was higher in larger exhibits with a higher proportion of fenced boundaries, and a lower proportion of visitor access. Females also exhibited higher concentrations at institutions with higher numbers of both males and females, and when housed in the same enclosure as other females only part of the time. However, fGCM was lower in female housed nearby to conspecific males, with the potential for auditory, visual and olfactory contact. Overall, males had lower fGCM when housed in walled enclosures, but in those where walls were present, a positive correlation was observed with increasing fGCM with a higher percentage of solid walls. Males kept on-show to the public exhibited higher fGCM concentration than those kept off-show. Male fGCM was also positively correlated with the number of females at an institution and higher in males housed with females only part of the time compared to those that were not housed with females at all. However, these differences in adrenal activity relating to social and environmental variables were not associated with differences in reproductive success, and furthermore, there were no differences in fGCM between proven and non-proven males or females, or between individuals that had reproduced during the last seven years. Although adrenal activity does correlate with certain aspects of the captive environment, it does not appear that chronic stress is having a detrimental effect on reproduction in this population.

In males, faecal testosterone metabolite concentration was also correlated with certain aspects of the social and physical environment. Males kept on show and those with no opportunity to escape from view exhibited higher fTt concentration, as did those in smaller enclosures with a higher proportion of solid walls, and a lower proportion of fencing surrounding their enclosure. Males also had higher fTt when housed with females during oestrus compared to not at all, and when housed with visual, auditory and olfactory contact of females.

In summary, although faecal glucocorticoid and testosterone metabolite concentration were correlated with certain aspects of the captive environment, individuals were no more likely to breed under particular social or environmental conditions. Furthermore, non-proven breeders did not have higher fGCM concentration than proven-breeders, indicating that chronic stress may not be a factor in reduced reproductive success in this population.

6.1. Introduction

Ex situ populations of black rhinoceros fulfil an important conservation role for this critically endangered species, raising awareness of the species' plight, whilst acting as a genetic reserve for remaining wild populations. However, the growth of captive populations in America have been limited by high rates of mortality and inconsistent reproduction (Carlstead and Brown 2005; Carlstead et al. 1999a; Carlstead et al. 1999b; Foose and Wiese 2006; Roth 2006), while in Europe, sub-optimal rates of reproduction and high reproductive skew are limiting both growth and genetic potential (Chapter 4). In Chapter 5 of this thesis, intrinsic differences in reproductive hormones were observed between proven and non-proven breeders, both in terms of irregular oestrous cycles in females, and in testosterone concentration in males. It is therefore essential to investigate whether these differences observed between proven and non-proven breeders, could be related to extrinsic factors relating to the captive environment.

One potential avenue to investigate to help understand differences in reproductive success is the role of adrenal activity. Activation of the hypothalamic-pituitary-adrenal (HPA) axis, and the resulting production of glucocorticoids from the adrenal gland is one way in which the body responds to challenges in the environment, facilitating the mobilisation of energy stores to allow the body to respond accordingly to potential stressors (Moberg and Mench 2000). Although this is primarily an adaptive process, prolonged exposure to potential stressors can lead to chronic stress and the disruption of other processes, including reproduction, as resources are diverted elsewhere (Moberg and Mench 2000). Furthermore, products of the HPA axis including corticotropin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and glucocorticoids can directly inhibit the hypothalamic-pituitary-gonadal (HPG) axis, resulting in disruption of reproduction (Kalantaridou et al. 2004). Due to the inhibitory nature of these products on reproductive function, even relatively mild stressors at certain crucial times in the reproductive cycle can be disruptive (Moberg and Mench 2000).

Animals in captivity are faced with a number of potential stressors, to which they must respond on a daily basis (reviewed in Morgan and Tromborg (2007)), related to the often unnatural social and physical conditions under which they are maintained. For example, due to the limited space in captivity, enclosures are often not reflective of natural home-range sizes, particularly in large species such as the black rhinoceros.

Individuals may be maintained at higher densities than would normally be found *in situ*, often housed together with little or no opportunity to retreat. These factors have been shown to lead to stress-related behaviours in other species, such as stereotypic pacing (Clubb and Mason 2003; Clubb and Mason 2007), increased aggression (Descovich et al. 2012), and undesirable behavioural traits such as timidity and self-directed behaviours (Hansen and Berthelsen 2000). Another aspect of the captive environment that can potentially be perceived as a stressor is that of human presence, either in the form of keeping staff or visitors to zoological institutions. Although positive keeper-animal relationships can be beneficial (Waitt et al. 2002), forced interaction with humans can lead to the expression of negative behaviours (Mallapur and Chellam 2002) and the restriction of natural behaviours (Wood 1998), as well as a physiological stress response (Davis et al. 2005), and potentially lowered survivorship (Carlstead et al. 1999a). Furthermore, an un-naturalistic environment may contain or lack other elements important to well-being, such as a lack of natural enrichment (Waiblinger and Konig 2004), unnatural climatic conditions (Rees 2004), sounds (Carlstead et al. 1999a), odours of conspecifics or predatory species (Buchanan Smith et al. 1993); all of which could impact how well an individual copes with their environment. Individuals may also cope with these potential challenges differently (Sapolsky 1994), which can impact their vulnerability to disruption and adverse consequences on welfare, reproduction and behaviour.

Indeed, previous studies have found certain elements of the captive environment to be related to welfare and reproductive success in the black rhinoceros. Carlstead et al. (1999a; 1999b) investigated the role of the social and physical environment, and of individual behaviour on reproductive success in captive black rhinos in America. Reproductive success was increased when male rhinos were behaviourally subordinate to their female mate, whereas females had greater reproductive success when they scored lower on potentially negative behaviours such as chasing and stereotypies. These behaviours were also correlated with certain aspects of the captive environment, such as enclosure size, which was positively correlated with female reproductive success and negatively correlated with male dominance scores. Additionally, the percentage of the rhino's enclosure that was surrounded by high concrete walls and visitor viewing access were positively correlated with stereotypy in females and institutional mortality respectively. Together these results suggest that both social and physical elements of a black rhino's environment can influence how well they cope with captivity.

Carlstead and Brown (2005) then went on to investigate the role of adrenal activity in these relationships, and found that faecal glucocorticoid metabolite (fGCM) concentration was also higher in individuals where a greater proportion of their enclosure perimeter was accessed by visitors. Social factors were also associated with fGCM concentration, with higher variance observed in breeding pairs kept together outside of oestrus, and in pairs that exhibited higher rates of fighting. Differences in adrenal activity were also reflected in potential fitness measures; although reproductive success was not found to correlate with fGCM in males or females (Brown et al. 2001; Carlstead and Brown 2005), institutional mortality was associated with a higher variance in fGCM concentration. However, higher variance in fGCM has been observed in female white rhinos that were categorised as non-cycling (Carlstead and Brown 2005). This highlights the need for the role of the social and physical environment on adrenal activity to be investigated further in this species, to determine whether differences in reproductive success could be related to adrenal activity and unequal reproduction in this population.

Alternatively, the captive environment may have consequences on reproduction aside from adrenal activity. For instance, certain social or physical conditions may be more favourable for reproduction, either due to different environmental conditions making introductions more successful, or lead to more natural behaviour once rhinos have been introduced. The presence of conspecifics may also have a stimulatory effect on reproduction. As demonstrated by Christensen et al (2009), male testosterone concentration may be influenced by access to conspecifics, with higher testosterone concentration observed in males either housed with other males or with females, compared to those in isolation. As higher testosterone concentration has been observed in proven males in this population (Chapter 5), it is important to determine whether proven males also have more social stimulation, or whether other environmental conditions may be correlated with testosterone and reproductive success in males.

It is therefore important to investigate whether differences in the social and physical environment could be related to differential reproductive success in captive black rhinoceros, either via adrenal activity, or whether some conditions may be more favourable to enhance breeding opportunities. The aim of this chapter was to perform an exploratory analysis to investigate whether any extrinsic factors relating to captivity may be associated with reproductive success. Firstly, to determine 1) whether any

elements of the physical or social environment, or 2) any differences in temperament that may affect an individual's response to these factors, may be related to adrenal activity. If adrenal activity were related to some aspect of the captive environment, and if individuals differ in how they perceive or respond to such challenges in their environment, perhaps this could be related to the differential reproductive success observed within this population. Furthermore, in males, these same extrinsic factors were investigated in relation to testosterone concentration, to determine 3) whether extrinsic factors could explain why breeding males exhibit higher concentrations than non-breeding males. Finally, in both males and females extrinsic factors were compared between breeding and non-breeding individuals, and between those that had bred during the last seven years with those that had not, to determine 4) whether certain elements of the captive environment may be correlated with better reproductive success in captivity.

6.2. Methods

6.2.1. Study population

This study included 63 eastern black rhinos situated at 13 zoological institutions across Europe (Table 6.1), and consisted of 24 males between the ages of 2y 10m and 32y 6m and 39 females between the ages of 1y 3m and 40y 9m. However, complete questionnaire data were not available for 3 males and 3 females where faecal samples were collected, and faecal samples were not available for 1 male where questionnaire data was collected.

The reproductive history of each individual was determined from the EAZA studbook, and individuals were categorised as follows. Firstly, individuals were categorised by their age, with females between the ages of 5-32 and males between the ages of 7-32 considered to be of breeding age; females aged under five and males under seven were classed as immature, and individuals aged 33 and over classes as being post-reproductive. Those individuals in the reproductive age class (n=17 males; n=31 females) were then further categorised as proven breeders if they had ever produced a live calf by the end of 2010 (premature births were not considered for this purpose, in case pregnancy loss could be a factor in poor reproductive success), whereas those that had never produced a live calf were considered non-proven. However, to distinguish between individuals that were currently breeding and those that may have bred

previously but have not reproduced for some time, a further category was established that included individuals that had not produced a calf for more than 7 years. The average inter-birth interval in this population is around 3 ½ years, so this timescale represents double the period in which a female would ideally have produced a subsequent calf. Therefore breeding age individuals were also categorised as either 1) proven breeders that had produced a calf within the last 7 years, proven breeders that had not produced a calf within the last 7 years, and non-proven individuals.

Table 6.1: Summary of females from which faecal samples and questionnaire data were collected as part of the study, including their age and reproductive category during the period of sample collection.

SB #	Name	Location [a]	Sex	Age [b]	Breeding status [c]	Breeding status (last 7 years) [d]	Faecal samples	Questionnaire
384	Rosie	Chester	F	21.1	NP	NP	✓	✓
680	Kitani	Chester	F	13.6	P	P<7	✓	✓
696	Manyara	Chester	F	12.4	NP	NP	✓	✓
883	Zuri	Chester	F	5.0	Y		✓	
898	Ema Elsa	Chester	F	8.2	P	P<7	✓	✓
947	Malindi	Chester	F	6.1	NP	NP	✓	✓
956	Bashira	Chester	F	3.1	Y		✓	✓
532	Tisa	Doué la Fontaine	F	16.0	NP	NP	✓	✓
910	Binti	Doué la Fontaine	F	7.2	NP	NP	✓	✓
387	Jessi	Dvur Kralove	F	25.4	P	P<7	✓	✓
619	Elba	Dvur Kralove	F	13.7	P	P<7	✓	✓
685	Jola	Dvur Kralove	F	12.6	P	P<7	✓	✓
689	Jane Lee	Dvur Kralove	F	12.3	NP	NP	✓	✓
876	Maischa	Dvur Kralove	F	4.4	Y		✓	✓
878	Etosha	Dvur Kralove	F	3.7	Y		✓	✓
417	Sany	Hannover	F	20.5	P	P>7	✓	✓
436	Sabah	Hannover	F	19.1	P	P>7	✓	✓
754	Rufiji	Howletts	F	10.9	NP	NP	✓	✓
762	Salome	Howletts	F	10.3	NP	NP	✓	✓
437	Nane	Krefeld	F	20.7	P	P<7	✓	✓
295	Mana	Magdeburg	F	28.5	P	P<7	✓	
559	Maleika	Magdeburg	F	14.2	NP	NP	✓	
428	Sita	Paignton	F	21.1	P	P<7	✓	✓
454	Siwa	Pont Scorff	F	18.8	NP	NP	✓	✓
195	Rukwa	Port Lympne	F	40.8 [e]	P	P>7	✓	✓
342	Arusha	Port Lympne	F	27.8	P	P>7	✓	✓
408	N'akuru	Port Lympne	F	21.5	P	P>7	✓	✓
455	Etna	Port Lympne	F	19.0	P	P<7	✓	✓
456	Jaga	Port Lympne	F	18.1	P	P>7	✓	✓
558	Vuyu	Port Lympne	F	19.8	P	P<7	✓	✓
663	Ruaha	Port Lympne	F	14.3	P	P<7	✓	✓
879	Zawadi	Port Lympne	F	4.3	Y		✓	✓
880	Grumeti	Port Lympne	F	4.3	Y		✓	✓
888	Solio	Port Lympne	F	9.7	NP	NP	✓	✓
911	Nyasa	Port Lympne	F	8.4	NP	NP	✓	✓
950	Damara	Port Lympne	F	5.3	NP		✓	✓
968	Nyota	Port Lympne	F	1.3	Y		✓	✓
662	Wanda	Zurich	F	14.3	NP	NP	✓	✓
861	Samira	Zurich	F	9.6	NP	NP	✓	✓

[a] current location when samples were collected for study; [b] age at the end of the sample collection period; [c] P=Proven – has produced a live offspring, NP=Non-proven – has never produced a live offspring; [d] P<7=Proven and has bred during last 7 years, P>7=Proven but not bred during last 7 years, NP=Non-proven – has never produced a live offspring; [e] estimated date of birth.

Table 6.2: Summary of males from which faecal samples and questionnaire data were collected as part of the study, including their age and reproductive category during the period of sample collection.

SB #	Name	Location [a]	Sex	Age [b]	Breeding status [c]	Breeding status (last 7 years) [d]	Faecal samples	Questionnaire
714	Magadi	Chester	M	12.8	P	P<7	✓	✓
750	Sammy	Chester	M	12.0	P	P<7	✓	✓
453	Kata Kata	Doué la Fontaine	M		NP	NP		✓
955	Asani	Chester	M	2.8	Y		✓	✓
268	Isis	Dvur Kralove	M	32.5	P	P<7	✓	✓
283	Jimm	Dvur Kralove	M	31.2	P	P<7	✓	✓
483	Baringo II	Dvur Kralove	M	17.5	NP	NP	✓	✓
659	Mweru	Dvur Kralove	M	13.7	NP	NP	✓	✓
877	Davu	Dvur Kralove	M	4.2	Y		✓	✓
926	Dzanti	Dvur Kralove	M	2.5	Y		✓	
927	Thabo	Ebeltoft	M	4.3	Y		✓	✓
928	Kito	Ebeltoft	M	4.4	Y		✓	✓
349	Kifaru II	Hannover	M	27.9	P	P>7	✓	✓
890	Vungu	Howletts	M	8.4	NP	NP	✓	✓
533	Taco	Koln	M	15.5	NP	NP	✓	
528	Usoni	Krefeld	M	15.8	P	P<7	✓	✓
653	Madiba	Magdeburg	M	20.1	P	P<7	✓	
892	Manyara	Paignton	M	8.6	NP	NP	✓	✓
438	Jakob	Pont Scorff	M	19.5	NP	NP	✓	✓
341	Kingo	Port Lympne	M	27.5	P	P<7	✓	✓
430	Quinto	Port Lympne	M	20.4	P	P>7	✓	✓
903	Zambezi II	Port Lympne	M	8.3	NP	NP	✓	✓
951	Monduli	Port Lympne	M	5.3	Y		✓	✓
857	Jeremy	Zurich	M	9.8	NP	NP	✓	✓

[a] current location when samples were collected for study; [b] age at the end of the sample collection period; [c] P=Proven – has produced a live offspring, NP=Non-proven – has never produced a live offspring; [d] P<7=Proven and has bred during last 7 years, P>7=Proven but not bred during last 7 years, NP=Non-proven – has never produced a live offspring; [e] estimated date of birth.

6.2.2. Faecal sample collection and preparation

Faecal samples collected and analysed in Chapter 5 were also used for this study. This included a total of 4048 faecal samples (1075 male samples and 2973 female samples) collected at least weekly from males and non-pregnant females, over a period of between 4 and 12 months. In 27 individuals (9 males and 18 females), samples were collected for over 12 months, to rule-out any seasonal differences in fGCM concentration. Samples were collected by keepers as soon as possible after defecation, taking multiple sub-sections from different areas of the faecal bolus to allow for potential uneven distribution within the sample, and combined in a zip-lock plastic bag. Samples were then frozen at -20°C following collection, and stored before shipment to Chester Zoo, UK for analysis.

Hormone metabolites were extracted from faecal samples according to an established wet-weight shaking extraction method (Edwards et al. 2013; Walker et al. 2002) (see Chapter 2 section 2.2.3 and Appendix 1 for detailed description of methods and protocols respectively). In brief, each sample was thawed, thoroughly mixed and weighed (0.5g±0.003g), before adding 5ml 90% methanol, vortexing and shaking overnight on an orbital shaker. Each sample was then vortexed and centrifuged for 20 minutes at 598g. The supernatant was decanted, dried under air, re-suspended in 1ml 100% methanol and the resulting faecal extract stored at -20°C until analysis.

6.2.3. Enzyme immunoassay

A previously described enzyme immunoassay was used to measure faecal glucocorticoid metabolites ((Watson et al. 2013), adapted from Munro and Stabenfeldt, (1984)) (see Chapter 2 section 2.2.4 and Appendix 1 for detailed description of methods and technique protocols respectively). The EIA utilised a polyclonal antiserum raised against corticosterone (CJM006; C.J. Munro, University of California, Davis); corresponding horseradish peroxidase (HRP) conjugated label (C.J. Munro, University of California, Davis); and standards (Sigma-Aldrich, UK) on a Nunc-Immuno Maxisorp (Thermo-Fisher Scientific, UK) microtitre plate. Black rhino faecal extracts were diluted as necessary in EIA buffer (female 1:20 and male 1:20), and run in duplicate (50µl) on the EIA.

6.2.4. Biochemical validation

The CJM006 EIA was biochemically validated for measuring glucocorticoid metabolites in 1) male and 2) female black rhino faecal extract through parallelism 1) R_2=0.987, $F_{1,7}$=537.761, P<0.001 and 2) R_2=0.982, $F_{1,7}$=377.007, P<0.001, and matrix interference assessment 1) R_2=0.995, $F_{1,7}$=1471.256, P<0.001 and 2) R_2=0.999, $F_{1,7}$=7133.701, P<0.001 (see Chapter 2, section 2.4 for full details). Intra- and inter-assay CVs were 12.9%, 7.2% and 8.5% for high and low binding synthetic and biological controls respectively. The cross reactivities for this antiserum have been described elsewhere (Watson et al. 2013)(See Appendix 2 for full details).

6.2.5. Questionnaire

Information about each individual was collected using questionnaires sent to each institution. These questionnaires were designed to collect information about each individual to coincide with sample collections for faecal hormone analysis, to investigate whether any factors were associated with reproductive success or differences in hormone metabolite concentrations. The use of multi-institutional surveys of behavioural indicators based on keeper assessment has been well validated in a number of species (Shepherdson and Carlstead 2001; Shepherdson et al. 2004; Whitham and Wielebnowski 2009), which allow the keepers that work with animals on a daily basis to score individuals on how they typically respond to a number of different scenarios. Carlstead et al. (1999b) used keeper ratings to assess behaviour profiles in captive black rhinoceros in US zoos, and deemed them a reliable and valid cross-institutional tool to investigate differences between black rhinoceros.

Firstly, keepers that work with the rhinos on a daily basis were asked to complete a number of ratings regarding their behaviour (Table 6.3; based on (Carlstead et al. 2000)), to determine whether underlying differences in temperament may affect their response to potential challenges, or influence reproductive success. Keepers at each institution were asked to score each rhino on a 5 point scale, reflecting how often or how likely the rhinos were to express certain behaviours. Behaviours of interest included how often an individual was seen pacing, how likely an individual was to approach keepers, how they react to their environment, such as their response to

unexpected events or new surroundings, and how changeable their temperament is over time.

Secondly, information regarding each individual's social and physical environment was recorded. This included the number of conspecifics in close proximity, whether these were males or females, and whether they were housed in the same enclosure, neighbouring enclosure so that they could see, hear, smell and/or touch one another (Table 6.4). Finally, information was also collected relating to the size and design of the rhino's enclosure (Table 6.5), including aspects such as area, materials used to construct boundary walls, and visitor access to the rhino's indoor and/or outdoor enclosure.

Table 6.3: Descriptions of behaviours given to keepers at each institution, who were asked to score the animals in their care on a scale of 1-5 (or 1-3 for 'changeable'), and were asked to consider how often a particular behaviour was expressed, or how each individual rhino would behave most of the time.

	1	**2**	**3**	**4**	**5**
How often are the following behaviours expressed:					
Pacing	Daily	Weekly	Monthly	Few times in lifetime	Never
Charging	Daily	Weekly	Monthly	Few times in lifetime	Never
Behaviour towards keepers:					
Approach keepers	Spontaneously approaches		Needs encouragement		Keeps Distance
Seek contact from keepers	Seeks contact		Tolerates contact		Avoids contact
Interested in keeper activity	Curious/investigates		Attentive but keeps distance		Uninterested
Nervous around keepers	Calm		Sometimes uneasy		Anxious
Approach new people	Readily approaches		Hesitant		Avoids
Aggressive towards people	Not at all		Snorts		Charges
Behaviour towards environment:					
Watchful of surroundings	Always vigilant		Sometimes observant		Non-responsive
Curious of new objects/surroundings	Curious, readily explores		Explores with caution		Uninterested
Approach novel objects/surroundings	Bold		Hesitant		Avoids
Nervous within environment	Relaxed		Sometimes uneasy		Anxious
Response to unexpected events	Calm		Slightly disturbed		Agitated
Active/explore environment	Very active		Quite active		Inactive
Startled by sudden sounds/movements	Very easily		Sometimes		Never
How changeable is this individual's behaviour:	Almost always behaves the same	Sometimes can be unpredictable	Very unpredictable	-	-

Table 6.4: Questions used to collect information on social aspects of a rhino's environment, given to keepers at each institution, and answered separately for each individual included in the study.

	1	**2**	**3**	**4**	
Has this individual been housed in the same enclosure as other individual(s) of the same sex?	Not at all	Some of the time	All the time		
Has this individual been housed in the same enclosure as other individual(s) of the opposite sex?	Not at all	During oestrus only	Some of the time, but not limited to oestrus	All the time	
Are any other rhinos kept nearby?					
Number of males housed in adjacent enclosure - physical contact	0	1	2	3	>3
Number of males housed nearby, but not adjacent - visual/auditory/olfactory contact	0	1	2	3	>3
Number of females housed in adjacent enclosure - physical contact	0	1	2	3	>3
Number of females housed nearby, but not adjacent - visual/auditory/olfactory contact	0	1	2	3	>3

Table 6.5: Questions used to collect information on physical aspects of a rhino's environment, given to keepers at each institution, and answered separately for each individual included in the study.

	1	2	3	4	5	6
What is the approximate area of this individual's outdoor enclosure?	<1000 m^2	1000 – 2000 m^2	2000 – 5000 m^2	> 5000 m^2		
Which of the following materials are used for the enclosure boundaries?						
Solid wall (*visual barrier*)	None	0-20%	20-40%	40-60%	60-80%	80-100%
Fence & vegetation (*visual barrier*)	None	0-20%	20-40%	40-60%	60-80%	80-100%
Fence only (*not a visual barrier*)	None	0-20%	20-40%	40-60%	60-80%	80-100%
When the zoo is open, is this rhino kept on show to the public?	Yes	No				
When the zoo is open, how much of the enclosure perimeter can visitors access to within 10m?	None	0-20%	20-40%	40-60%	60-80%	80-100%
When the zoo is open, do visitors have access to indoor enclosures?	Yes	No				
When the zoo is open, does this rhino have the opportunity to escape from view?						
By going inside	Yes	No				
Behind barriers and/or structures	Yes	No				

6.2.6. Data analysis

In order to limit the number of questionnaire variables used for analyses, any categories from the questionnaire were excluded where there was little or no variation between individuals, or where there were a limited number of responses within a particular category (i.e. behaviour rarely seen or conditions were non-applicable in the majority of responses). To investigate the effect of extrinsic factors on faecal hormone metabolite concentration, weekly samples were analysed for faecal glucocorticoid (fGCM; males or females, analysed separately) or testosterone (Tt; males only) metabolite concentration (n=1075, 3079 and 1455 respectively), and compared using generalised linear mixed models (GLMM's) in MLwiN version 2.02 (Rasbash et al. 2005). Environmental, social and behavioural factors were analysed across all individuals for their relationship with fGCM; for testosterone differences according to reproductive category, only social and environmental factors within mature males were investigated. Normality tests were first conducted in IBM® SPSS® statistics version 20, and hormone data were transformed where necessary, using $\log_{10}$ transformations to improve the distribution of data ($\log_{10}$ GC and $\log_{10}$ Tt).

As faecal samples were collected repeatedly within multiple individuals and across multiple institutions, date of sample collection, subject ID and institution were fitted as nested random effects in all models, to control for non-independence of data (Bolker et al. 2009). Questionnaire data were then used individually as fixed effects to explore the explanatory effect of each extrinsic factor on hormone metabolite concentration. Either categorical or continuous fixed effects were incorporated into the GLMM, with categorical variables assigned a reference category to which all other categories were compared, with post-hoc comparisons between each group when necessary. Additionally, to control for previously observed differences in testosterone concentration with reproductive category (Chapter 5), in addition to the main effects of each of the extrinsic factors, interactions with reproductive category were assessed in all models of $\log_{10}$ Tt, and are reported where relevant.

Although a multivariate approach was also used to investigate the influence of social, environmental or behavioural factors on faecal hormone metabolite concentration (multiple variables from a single category combined into a GLMM), these models were not robust. Instead, individual relationships between each fixed effect and either $\log_{10}$ GC or $\log_{10}$ Tt are reported, taking into account the repeated measures within

individuals. A normal error structure was used for all models of $\log_{10}$ hormone metabolite concentration, and the significance of each fixed effect was determined using the Wald statistic and chi-squared (χ^2) distribution, with alpha set to 0.05.

Additionally, questionnaire variables were used to investigate reproductive success, according to reproductive category (proven versus non-proven; and bred during the last 7 years, not bred during the last 7 years, or never bred). As many of the questionnaire variables were ratings, and therefore were generally not normally distributed (as determined using Kolmogorov Smirnov normality tests also conducted in SPSS® version 20), comparison of variables across reproductive categories were analysed using Mann Whitney U tests (for comparing two groups) or Kruskal Wallis (comparing three or more groups), both conducted in IBM® SPSS® statistics version 20. Additionally, the relative proportions of categorical extrinsic variables according to reproductive categories were investigated using cross-tabulation with Pearson's chi-square test, in IBM® SPSS® statistics version 20.

6.3. Results

6.3.1. Glucocorticoids and breeding status

Using all faecal samples analysed for fGCM from non-pregnant reproductive-age females across the study period, there was no difference in $\log_{10}$ fGCM concentration between proven and non-proven breeders (GLMM χ^2=1.266, df=1, P=0.26). Similarly, there was no difference between non-proven females and proven females that have bred during the last seven years (GLMM χ^2=0.339, df=1, P=0.56); between non-proven females and proven females that have not bred during the last seven years (GLMM χ^2=1.533, df=1, P=0.22), or between proven females that have bred during the last seven years or not (GLMM χ^2=0.502, df=1, P=0.48).

In males, $\log_{10}$ fGCM was predicted by age (GLMM χ^2=6.007, df=1, P=0.014) with lower fGCM concentration in males within younger age classes (age 1-7 vs. 17-33 (GLMM χ^2=8.660, df=1, P=0.003), and 7-17 vs. 17-33 (GLMM χ^2=7.744, df=1, P=0.005)). Among reproductive-age males, there were no differences in $\log_{10}$ fGCM between proven and non-proven breeders (GLMM χ^2=0.061, df=1, P=0.80). Furthermore, there were no differences according to whether males had sired offspring during the last seven years, with no difference between non-proven males and proven males that have bred during

the last seven years (GLMM χ^2=0.003, df=1, P=0.96); between non-proven males and proven males that have not bred during the last seven years (GLMM χ^2=0.444, df=1, P=0.51), or between proven males that have bred during the last seven years or not (GLMM χ^2=0.573, df=1, P=0.45).

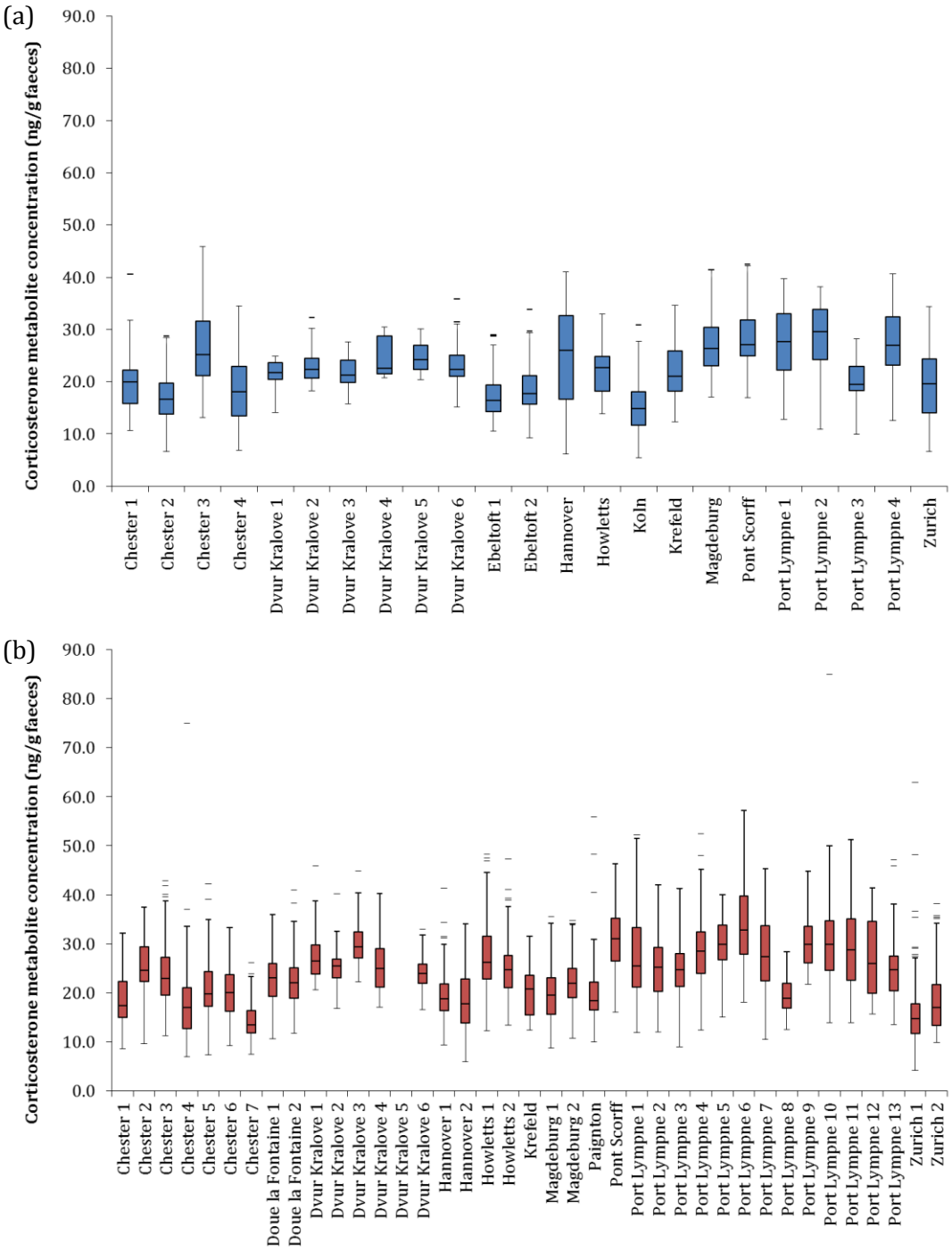

Figure 6.1: Boxplot of faecal glucocorticoid metabolite (fGCM) concentration in (a) male and (b) female black rhinos at different institutions across Europe. The shaded box represents the interquartile range (IQR, 25th and 75th percentile of the data); the line through the box represents the median value; lower and upper whiskers represent the minimum and either the maximum value or 1.5x IQR respectively; lines represent outliers (>1.5x IQR).

6.3.2. Glucocorticoids, environment and behaviour

Faecal glucocorticoid metabolite concentration was highly variable both within and between individuals (Figure 6.1), and between institutions (males: χ^2=113.508, df=10, P<0.001; females: χ^2=84.925, df=10, P<0.001).

In both males and females, certain aspects of enclosure design were related to $\log_{10}$ fGCM concentration (details provided in Table 6.6). In both males and females, individuals that were housed in enclosures with at least part of the boundary composed of solid walls, had lower $\log_{10}$ fGCM than those individuals where solid walls were not present. Of those males that had solid walls as part of their enclosure boundary, there was a positive correlation between the percentage of the boundary that was composed of solid walls and $\log_{10}$ fGCM concentration, meaning that a greater proportion of solid walls around their enclosure was associated with higher $\log_{10}$ fGCM concentration. However, this was not the case for females, as there was no correlation between percentage walls and $\log_{10}$ fGCM; instead, there was a positive correlation between the percentage of a female's enclosure surrounded by fencing and $\log_{10}$ fGCM concentration, so females with fencing around a higher proportion of their enclosure had higher $\log_{10}$ fGCM concentration. Additionally, in females but not in males, the area of an individual's enclosure was positively correlated to $\log_{10}$ fGCM, with higher $\log_{10}$ fGCM in females with larger enclosure area.

Three aspects of visitor access were also related to $\log_{10}$ fGCM. In males, rhinos that were kept on show to the public had higher $\log_{10}$ fGCM concentration than males that were kept off show to the public. However, in females, the relationships observed between visitor access and $\log_{10}$ fGCM were in the opposite direction. The percentage of the enclosure perimeter to which visitors could access to within 10m was negatively correlated with $\log_{10}$ fGCM, so higher $\log_{10}$ fGCM was associated with visitor access around a smaller proportion of the outdoor enclosure. Similarly, females housed in exhibits where visitors had access to their indoor enclosures had lower $\log_{10}$ fGCM concentration than females where visitors did not have access indoors.

The presence and proximity of other rhinos was also related to $\log_{10}$ fGCM concentration (details provided in Table 6.7). In females, $\log_{10}$ fGCM was positively correlated with the number of males, and the number of other females at an institution. In males, this correlation was also apparent for the number of females at an institution, but not with the number of other males. Furthermore, the number of rhinos housed

nearby was also related to $\log_{10}$ fGCM. Males that were housed with females some of the time, but not limited to oestrus also had higher $\log_{10}$ fGCM than males that were never housed in the same enclosure as females, but there were no differences between males that were housed with a female all of the time, or only during oestrus. Although only two males included in the study were housed with another male all the time, these two males had higher $\log_{10}$ fGCM than males that were never housed with another male. In females, there were no significant differences between females that were housed in the same enclosure with males all or part of the time, but female housed with other females some of the time had higher $\log_{10}$ fGCM than females that were never housed with other females. Additionally, $\log_{10}$ fGCM were lower in females that were housed nearby to males, so that they could potentially see, hear, and smell one another, but did not have physical contact.

Individual differences in behaviour were also related to $\log_{10}$ fGCM (details provided in Table 6.8). Although there were no correlations apparent in either males or females between adrenal activity and stereotypic or negative behaviours such as pacing or charging, keeper ratings of how individuals respond to their environment did vary with $\log_{10}$ fGCM. In particular, both males and females exhibited differences in $\log_{10}$ fGCM according to how changeable keepers rated their behaviour to be over time. Rhinos that were scored as almost always behaving the same had lower $\log_{10}$ fGCM than rhinos that were scored as sometimes unpredictable (in females, and a tendency in males), or very unpredictable in their behaviour (both males and females). When scored on how likely individuals were to express certain behaviours towards keepers, females that were deemed to be more interested in keeper activity and more aggressive towards people all had higher $\log_{10}$ fGCM concentration than individuals that were scored lower on those aspects of their behaviour. In males, there were no correlations between $\log_{10}$ fGCM and behaviour towards keepers. However, when keepers scored their rhinos on their behaviour towards environmental aspects, males that were scored as less agitated by unexpected events, but more easily startled by sudden sounds/movements also had higher $\log_{10}$ fGCM. Females that were scored as bolder around novel objects or surroundings, and more reactive to sudden sounds and movements, also tended to have higher $\log_{10}$ fGCM concentration than other females that did not score so highly.

Table 6.6: Environmental factors related to log_{10} fGCM concentration in male and female black rhinoceros (see Table 6.5 for description of environmental variables). Each fixed effect was entered individually into a GLMM, to obtain the effect of that component of the captive environment on log_{10} fGCM concentration; the GLMM includes random effects to control for multiple faecal samples collected within multiple individuals at multiple institutions. Multivariate models were not robust, and are not reported. Table shows the magnitude and direction of the relationship between each fixed effect and the dependent variable of log_{10} fGCM (effect size and corresponding standard error) and significance of associated test statistic (Wald statistic) for each potential explanatory variable.

	Males						Females					
Fixed effect	**Relationship**	**Effect size**	**SE**	**Wald statistic**	**df**	**P**	**Relationship**	**Effect size**	**SE**	**Wald statistic**	**df**	**P**
Enclosure boundary: *Solid walls included in enclosure boundary* (†*Yes*)	Yes < No	0.067	0.030	5.048	1	**0.025**	Yes < No	0.112	0.022	26.378	1	**<0.001**
% Solid walls in enclosure boundary	Positive	0.027	0.013	4.244	1	**0.039**	-	0.008	0.014	0.292	1	0.59
% Fence in enclosure boundary	-	0.010	0.011	0.867	1	0.35	Positive	0.017	0.009	3.925	1	**0.048**
Enclosure area (m^2):	-	-0.002	0.015	0.016	1	0.90	Positive	0.032	0.012	6.871	1	**0.009**
Visitor access: *% Perimeter of outdoor enclosure has visitor access*	Negative	-0.020	0.012	2.790	1	***0.095***	Negative	-0.042	0.007	31.645	1	**<0.001**
On-show vs. off-show†	On-show > off-show	0.123	0.032	15.167	1	**<0.001**	-	0.050	0.041	1.508	1	0.22
Visitor access to indoor enclosures (†*No*)	-	0.052	0.038	1.896	1	0.17	Access < No access	-0.060	0.031	3.837	1	**0.050**
Opportunity to escape from view (†*Yes*)	-	0.029	0.038	0.563	1	0.45	-	-0.055	0.037	2.145	1	0.14

† Denotes reference category for categorical variables

P values in bold represent those significant at the 0.05 level, tendencies (P<0.10) are denoted by bold italics.

Table 6.7: Social factors related to log_{10} fGCM concentration in male and female black rhinoceros (see Table 6.4 for description of social variables). Each fixed effect was entered individually into a GLMM, to obtain the effect of that component of the social environment on log_{10} fGCM concentration; the GLMM includes random effects to control for multiple faecal samples collected within multiple individuals at multiple institutions. Multivariate models were not robust, and are not reported. Table shows the magnitude and direction of the relationship between each fixed effect and the dependent variable of log_{10} fGCM (effect size and corresponding standard error) and significance of associated test statistic (Wald statistic) for each potential explanatory variable.

	Males						Females				
Fixed effect	**Relationship**	**Effect size**	**SE**	**Wald statistic**	**df**	**P**	**Relationship**	**Effect size**	**SE**	**Wald statistic**	**df**	**P**
No. rhinos at institution:												
Males		0.011	0.008	2.051	1	0.15	Positive	0.026	0.006	15.980	1	**<0.001**
Females	Positive	0.007	0.003	4.749	1	**0.029**	Positive	0.011	0.003	16.619	1	**<0.001**
Housed with other rhinos:												
Housed with same sex										5.885	2	***0.053***
†*Not at all*												
Some of the time		-	-	-	-	-	Some of the time > Not at all	0.077	0.034	5.170	1	**0.023**
All of the time	All of the time > Not at all	0.128	0.031	16.608	1	**<0.001**		0.049	0.032	2.324	1	0.13
Housed with opposite sex										1.701	3	0.64
†*Not at all*												
Some of the time, not limited to oestrus	Some of the time > Not at all	0.096	0.034	7.975	1	**0.005**		0.001	0.034	0.000	1	1.00
During oestrus only	-	0.053	0.048	1.229	1	0.27		-0.015	0.052	0.081	1	0.78
All the time	-	0.054	0.048	1.249	1	0.26		0.048	0.041	1.382	1	0.24
Housed near other rhinos:												
Housed near same sex (†*Yes*)	-	0.011	0.037	0.094	1	0.76		-0.004	0.056	0.004	1	0.95
Housed near opposite sex (†*Yes*)	-	-0.034	0.038	0.811	1	0.37	Yes < No	0.120	0.034	12.505	1	**<0.001**

† Denotes reference category for categorical variables, where 3 or more categories exist (Housed with other rhinos) the overall test statistic is also given in addition to pairwise comparisons. P values in bold represent those significant at the 0.05 level, tendencies (P<0.10) are denoted by bold italics.

Table 6.8: Ratings of individual behaviour (see Table 6.3 for description of behavioural traits) related to log_{10} fGCM concentration in male and female black rhinoceros. Fixed effects were either entered individually into a GLMM (pacing, charging and changeable), or were used to create a minimal multivariate model (behaviour towards keepers or environment), to obtain the effect of behaviour on log_{10} fGCM concentration; the GLMM includes random effects to control for multiple faecal samples collected within multiple individuals at multiple institutions. Table shows the magnitude and direction of the relationship between each fixed effect and the dependent variable of log_{10} fGCM (effect size and corresponding standard error) and significance of associated test statistic (Wald statistic) for each potential explanatory variable.

	Males						Females					
Fixed effect	**Relationship**	**Effect size**	**SE**	**Wald statistic**	**df**	**P**	**Relationship**	**Effect size**	**SE**	**Wald statistic**	**df**	**P**
Pacing	-	0.000	0.012	0.000	1	1.000		0.000	0.009	0.002	1	0.96
Charging	-	0.009	0.014	0.433	1	0.51		-0.011	0.011	1.010	1	0.31
Behaviour towards keepers:												
Approach keepers [a]	-	0.017	0.054	0.103	1	0.75		-0.003	0.017	0.038	1	0.85
Seek contact from keepers [a]	-	-0.009	0.020	0.187	1	0.67		0.000	0.023	0.000	1	1.00
Interested in keeper activity [a]	-	-0.002	0.016	0.013	1	0.91	Negative	-0.041	0.017	5.908	1	**0.015**
Nervous around keepers [a]	-	0.029	0.028	1.038	1	0.31		-0.001	0.022	0.002	1	0.96
Approach new people [a]	-	-0.018	0.025	0.516	1	0.47		-0.020	0.015	1.786	1	0.18
Aggressive towards people [a]	-	-0.028	0.022	1.665	1	0.20		0.020	0.010	4.049	1	**0.044**
Behaviour towards environment:												
Watchful of surroundings [b]	-	0.024	0.019	1.704	1	0.19		0.001	0.022	0.003	1	0.96
Curious of new objects/surroundings [b]	-	-0.005	0.016	0.101	1	0.75		-0.003	0.023	0.012	1	0.91
Approach novel objects/surroundings [b]	-	-0.004	0.020	0.045	1	0.83	Negative	-0.053	0.016	10.745	1	**0.001**
Nervous within environment [b]	-	-0.015	0.023	0.460	1	0.50		-0.008	0.017	0.238	1	0.63
Response to unexpected events [b]	Negative	-0.035	0.016	4.832	1	**0.028**		0.012	0.17	0.476	1	0.49
Active/explore environment [b]	-	-0.006	0.019	0.118	1	0.73		-0.004	0.015	0.071	1	0.79
Startled by sudden sounds/movements [b]	Negative	-0.052	0.024	4.485	1	**0.034**	Negative	-0.037	0.015	6.113	1	**0.013**

Continued….

Changeable †*Almost always behaves the same*				6.434	2	**0.040**				26.499	2	**<0.001**
Sometimes can be unpredictable	*> almost always behaves the same*	0.079	0.044	3.173	1	***0.07***	*> almost always behaves the same*	0.060	0.023	7.153	1	**0.007**
Very unpredictable	*> almost always behaves the same*	0.101	0.051	4.022	1	**0.045**	*> almost always behaves the same*	0.111	0.025	20.252	1	**<0.001**

[a,b] Variables with the same superscript letter were entered into the same GLMM, and non-significant terms subsequently removed until the minimal model was obtained. Non-significant terms were then re-entered individually, to determine their level of non-significance. All other variables were entered into GLMM alone.
† Denotes reference category for categorical variables, where 3 or more categories exist (Changeable) the overall test statistic is also given in addition to pairwise comparisons. P values in bold represent those significant at the 0.05 level, tendencies (P<0.10) are denoted by bold italics.

6.3.3. Testosterone and environmental factors

There were also relationships observed between male testosterone concentration and social and environmental factors (details provided in Tables 6.9 and 6.10). Log_{10} Tt was negatively correlated with both enclosure area and the percentage of fencing surrounding their enclosure, and positively correlated with the percentage of solid walls. Log_{10} Tt was also higher in males kept on show as opposed to off show, and was also higher in males that had no opportunity to escape from public view either by going inside or behind structures in their enclosure.

Faecal testosterone concentration was not correlated with the number of other males present, either in the same enclosure, housed nearby with the potential to see, hear and smell one another, or the total number of other males housed at an institution. There was also no correlation with the total number of females housed at an institution. However, males that were housed with females during oestrus had higher testosterone than males that were not housed in the same enclosure as females at all during the study period. This effect was also observed with females that were housed nearby, but not in the same enclosure. Furthermore, there was also a significant interaction between whether males were housed near the opposite sex and reproductive category, with proven males exhibiting higher testosterone concentration than non-proven males, even when housed near to females.

Table 6.9: Environmental factors related to faecal testosterone metabolite concentration (log_{10} Tt) in male black rhinoceros. Each fixed effect was entered individually into a GLMM, to obtain the effect of that component of the physical environment on log_{10} Tt concentration; the GLMM includes random effects to control for multiple faecal samples collected within multiple individuals at multiple institutions. Main effects and interactions were investigated, but for simplicity only significant or marginally significant interaction terms have been included. Multivariate models were not robust, and are not reported. Table shows the magnitude and direction of the relationship between each fixed effect and the dependent variable of log_{10} fTt (effect size and corresponding standard error) and significance of associated test statistic (Wald statistic) for each potential explanatory variable.

Fixed effect	Relationship	Effect size	SE	Wald statistic	df	P
Enclosure boundary:						
Solid walls included in enclosure boundary (†*Yes*)	-	-0.075	0.052	2.094	1	0.15
% Solid walls in enclosure boundary	Positive	0.065	0.024	7.349	1	**0.007**
% fence in enclosure boundary	Negative	-0.035	0.017	4.150	1	**0.042**
Enclosure area (m^2):	Negative	-0.055	0.021	6.763	1	**0.009**
Visitor access:						
% Perimeter of outdoor enclosure has visitor access	-	0.017	0.021	0.663	1	0.42
*Interaction: % perimeter*reproductive category* († *proven*)	Non-proven < proven	-0.032	0.017	3.658	1	***0.056***
On-show vs. off-show †	On show > Off-show	0.166	0.058	8.041	1	**0.005**
Interaction: On-show reproductive category* († *proven*)	Non-proven < proven	-0.080	0.043	3.511	1	***0.06***
Visitor access to indoor enclosures (†*No*)	-	0.055	0.062	0.780	1	0.38
Opportunity to escape from view (†*Yes*)	Yes < No	0.125	0.057	4.754	1	**0.029**

† Denotes reference category for categorical variables. P values in bold represent those significant at the 0.05 level, tendencies (P<0.10) are denoted by bold italics.

Table 6.10: Social factors related to faecal testosterone metabolite concentration (log_{10} Tt) in male black rhinoceros. Each fixed effect was entered individually into a GLMM, to obtain the effect of that component of the social environment on log_{10} Tt concentration; the GLMM includes random effects to control for multiple faecal samples collected within multiple individuals at multiple institutions. Main effects and interactions were investigated, but for simplicity only significant or marginally significant interaction terms have been included. Multivariate models were not robust, and are not reported. Table shows the magnitude and direction of the relationship between each fixed effect and the dependent variable of log_{10} fTt (effect size and corresponding standard error) and significance of associated test statistic (Wald statistic) for each potential explanatory variable.

Fixed effect	Relationship	Effect size	SE	Wald statistic	df	P
No. rhinos at institution:						
Males	-	0.001	0.012	0.007	1	0.93
Females	-	0.001	0.005	0.059	1	0.81
Housed with other rhinos:						
Housed with same sex						
†Not at all vs. All of the time	-	0.026	0.090	0.086	1	0.77
Housed with opposite sex						
†Not at all				6.371	3	***0.095***
Some of the time, not limited to oestrus	-	0.074	0.055	1.801	1	0.18
During oestrus only	During oestrus only > Not at all	0.187	0.076	6.065	1	**0.014**
All the time	-	0.048	0.076	0.396	1	0.53
Housed near other rhinos:						
Housed near same sex (*†Yes*)	-	0.015	0.064	0.053	1	0.18
Housed near opposite sex (*†Yes*)	Yes > No	-0.164	0.051	10.529	1	**0.001**
*Interaction: Housed near opposite sex*reproductive category* (*† proven*)	Non-proven < proven	-0.125	0.057	4.793	1	**0.029**

† Denotes reference category for categorical variables, where 3 or more categories exist (Housed with other rhinos) the overall test statistic is also given in addition to pairwise comparisons. P values in bold represent those significant at the 0.05 level, tendencies (P<0.10) are denoted by bold italics.

6.3.4. Breeding status, environment and behaviour

Comparing social, environmental or behavioural factors between males and females according to reproductive categories, there were no significant differences that may explain whether an individual has previously bred or not (Appendix 6). Proven and non-proven breeders did not tend to be scored differently on how likely they were to express certain behaviours, or how they respond to keepers or to changes within their environment. This was the case for both males and females, and whether comparing individuals based on their lifetime reproductive status (proven versus non-proven) or whether they have bred during the last seven years. Similarly, individuals kept under certain social or environmental conditions were no more likely to have bred, either in their lifetime or during the last seven years.

However, there were a number of potential relationships observed, which did not quite reach significance, but may be worth investigating further (see Appendix 6 for details). Firstly, females that are proven, but have failed to reproduce during the past seven years tended to be scored as more vigilant (P=0.08) than either non-proven females or proven females that had bred more recently. These same females (P>7 category) also tended to be found in exhibits with a higher percentage of solid walls surrounding their enclosure (P=0.067). Both non-proven females and females that had not bred for at least seven years also tended to be housed in exhibits where visitors did not have access to the indoor enclosure (P=0.076). Finally, proven females tended to be housed at institutions with a greater number of conspecific males (P=0.081) and females (P=0.081).

In males, there was a tendency for more proven males to be housed in enclosures with solid concrete walls, and non-proven males to be housed in enclosures without solid walls (P=0.062). However, of those non-proven males where solid walls did make up a portion of their enclosure boundary, a greater proportion of the boundary was solid walls (P=0.099). It seems that non-proven males either have no solid walls as part of their enclosure boundary (meaning the boundary was predominantly fence), or had a high proportion of solid walls. As previously mentioned, there were only two males that were housed together all of the time (both aged 8 years), and these males were as yet unproven. This small sample size meant that non-proven males tended to live with males all of the time (P=0.051), and these two males also had no females housed

nearby, leading to a tendency for males not housed near females to be non-proven (P=0.051).

6.4. Discussion

The results of the current study do not support the hypothesis that chronic adrenal activity of captive black rhinoceros is related to poor reproductive success in this population, with no differences in fGCM concentration between proven and non-proven males or females. Although, differences in fGCM were observed between individuals, and were associated with a number of social and environmental factors investigated in this study, these were not consistent with differences in reproductive success. Furthermore, the relationships observed between fGCM and environmental factors in particular, were not always consistent with previous findings.

Many aspects of the captive environment have the potential to be perceived as stressors, including unnatural aspects of exhibit design and unnatural social groupings (Morgan and Tromborg 2007). Previous research on the relationship between the captive environment and adrenal activity in the black rhinoceros highlighted a number of factors that may contribute towards elevated adrenal activity and elevated mortality in this species (Carlstead and Brown 2005; Carlstead et al. 1999a). In the American *ex situ* population of black rhinoceros, elevated glucocorticoid concentration was found to be related to the proportion of an individual's exhibit with visitor access around the perimeter (Carlstead and Brown 2005). Furthermore, this aspect of enclosure design was also positively correlated with institutional mortality rates (Carlstead et al. 1999a), indicating a potential fitness consequence to the increased fGCM associated with visitor access. However, in the current study, the opposite relationship was observed, with fGCM in females negatively correlated with the percentage of visitor access to within 10m of the enclosure. Although this result is not consistent with previous findings, it may be related to the other environmental factors indicated in this study. Within females in this population, fGCM was also positively correlated with enclosure area and the percentage of fencing around the exhibit, whilst negatively correlated with the percentage of solid walls included within the enclosure. These environmental variables themselves were correlated within females however, with larger enclosures tending to be mainly fenced, and smaller enclosures often having a higher proportion of solid walls.

It is important to distinguish between activation of the HPA axis that enables an individual to respond and cope with its environment, and abnormal adrenal activity that may have potential fitness consequences. Glucocorticoids are not only illustrative of potentially negative stress, but are primarily a response to any perceived challenge to which the body must respond. An increase in glucocorticoids has been demonstrated in response to both positive and negative stimuli (Buwalda et al. 2012), and glucocorticoids are typically elevated during exercise (Coleman et al. 1998; Koolhaas et al. 2011). Perhaps the increased fGCM concentration observed in females in larger enclosures may not necessarily be indicative of negative stress due to enclosure design, but may reflect increased activity in larger enclosures. This might also explain why fGCM were lower in walled enclosures compared to those with no walls, as they were generally smaller.

This study has also indicated that social relationships may play a role in adrenal activity in this species, with increased fGCM concentration observed in both males and females with increased numbers of the opposite sex present at the same institution, with females also influenced by the number of same sex conspecifics. Furthermore, glucocorticoids were also elevated in both males and females that were housed with the opposite sex for part of the time compared to those that were not, and in females the same correlation was also observed when housed nearby to males, with the potential for auditory, visual and olfactory contact, but not with physical contact. However, again these aspects of the social environment were not correlated with reproductive success, indicating that although social interactions influence adrenal activity, they do not necessarily have detrimental consequences on reproduction.

Additionally, faecal testosterone metabolite concentration was also investigated in relation to social and environmental variables, to determine whether the previously observed differences between proven and non-proven males (Chapter 5) may be related to extrinsic factors. However, although faecal testosterone metabolite concentration was correlated with certain aspects of the social and physical environment, the same relationships were not apparent between proven and non-proven males. Instead, males that were kept on show or with opportunity to escape from view exhibited higher fTt concentration, as did those in smaller enclosures with a higher proportion of solid walls, and a lower proportion of fencing surrounding their enclosure. Males also had higher fTt when housed with females during oestrus compared to not at all, and when housed with visual, auditory and olfactory contact of

females. Although this suggests that perhaps social stimulation may indeed be important for testosterone concentration (Christensen et al. 2009), there remained a difference between proven and non-proven males, regardless of whether females were present or not. This suggests that perhaps testosterone may be influenced by other intrinsic factors that are yet to be identified in this population.

Although a number of differences in fGCM have been observed related to the social and physical environment, none of these were consistent with differences in reproductive success. In both males and females included in this study, no differences in fGCM were apparent between individuals that have ever produced a live calf and those that have not (proven versus non-proven), or whether they have bred during the last seven years. It may be that the variation observed both within and between individuals is more representative of natural variation in adrenal activity in response to daily challenges, rather than chronic stress attributable to the captive environment. However, this does not completely rule out the possibility that adrenal activity has an influence on reproduction in this population.

Individuals may vary in how they respond to potential challenges, and understanding how captive animals respond to their surroundings can be beneficial in optimising breeding management and captive welfare (Tetley and O'Hara 2012). Research conducted on a wide range of species has revealed that individuals may respond to potential challenges in a consistent manner based on differences in coping style (Koolhaas et al. 1999), temperament (Martin and Reale 2008), or personality (Carere et al. 2010). Although the terminology varies between studies, the behavioural and physiological responses remain consistent. Proactive or bold individuals tend to be more aggressive, but exhibit a relatively low glucocorticoid response to a potential stressor, while reactive, or shy individuals commonly have a heightened glucocorticoid response but behaviourally may withdraw. These differences in response to a stimulus may also have consequences for the physiological costs involved, for example the two subtypes can be prone to different types of stress-related disease (Koolhaas et al. 1999), and may have differential reproductive success (Smith and Blumstein 2008).

In both males and females in this population, fGCM varied according to temperament, with rhinos scored by their keepers as more unpredictable exhibiting higher fGCM than rhinos scored as almost always behaving the same. Although differences between proven and non-proven breeders did not reach significance, there was some indication that non-proven females also tended to be scored as more unpredictable than proven

females. If individuals scored as more unpredictable either perceive stressors differently, or exhibit an altered adrenal response to stimuli, it is possible that they may be more susceptible to disruption. It may therefore be useful to investigate changes in adrenal activity within females during periods of regular and irregular cyclicity, to determine if short-term changes in fGCM, as opposed to average concentrations, may be useful in determining whether adrenal activity may be related to reproductive success in this population. When looking at acute challenges, due to the short-term nature of the HPA response, averaging fGCM concentration across multiple faecal samples may remove the variation of interest. It may therefore be beneficial to investigate fGCM concentration on a finer scale, investigating short-term changes within individuals, on days when key events of interest occur (Edwards et al. 2013), Trotter et al. *in prep*; Hill et al. *in prep*). To investigate this further, it is important to determine whether any hormone changes occur within females exhibiting different cycle types (see Chapter 7).

Although the aim of this study was to investigate the potential effect of a wide range of social, environmental and behavioural factors on faecal hormone metabolites and reproductive success, the number of variables investigated here could have had an influence on the outcome of these analyses. Perhaps reducing the number of variables of interest could be beneficial, both to minimise the risk of inflated type I errors from multiple testing, and to reduce the potential impact of correlated variables. This approach may improve the inferences that could be made regarding the influence of the social and physical environment on reproductive success and captive welfare. Additionally, the positive correlation between both fTt and fGCM and age in males should be taken into consideration when performing these analyses, to ensure that this is not confounding any potential relationships.

Understanding the potential factors that influence well-being and reproductive success is complex issue, with a range of elements that could potentially affect individuals within a captive setting, and variation in how individuals may respond to potential challenges they face. Although we found no differences in fGCM between proven and non-proven males or females, indicating that chronic stress may not necessarily be contributing to reduced reproductive success, it may be that individuals vary in how they perceive and respond to potential challenges in their environment, which could have consequences on their overall reproductive success.

6.5. Conclusion

- Although fGCM was variable between individuals, there were no consistent differences between breeding and non-breeding males or females, suggesting that chronic stress may not be contributing towards the reproductive skew observed in this population.
- However, certain aspects of the social and physical environment were related to adrenal activity in male and female black rhinos, including enclosure characteristics, visitor access and access to conspecifics.
- However, the results of this current study were inconsistent to those found previously in the American captive population of black rhinoceros, indicating that further investigation may be required to distinguish between normal variation in response to daily challenges and chronic adrenal activity that could have detrimental fitness consequences.
- Additionally, although faecal testosterone metabolite concentration in males was also correlated with certain aspects of the social and physical environment, these relationships were also inconsistent with reproductive history.
- Differences in reproductive success do not appear to be strongly correlated with extrinsic factors investigated here, which indicates that perhaps the differences observed in fecundity may be due to underlying differences between individuals, as opposed to elements of the captive environment strongly influencing whether or not an individual is likely to breed.

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- Paignton Zoo, Paignton, UK
- Port Lympne Wild Animal Park, Hythe, Kent, UK
- Howletts Wild Animal Park, Bekesbourne, Kent, UK
- Zoo Zürich, Zurich, Switzerland
- Zoologischer Garten Magdeburg, Magdeburg, Germany
- Zoo de Pont-Scorff, Pont-Scorff, France

CHAPTER 7:

7. INVESTIGATING ABNORMAL REPRODUCTIVE CYCLICITY IN FEMALE EASTERN BLACK RHINOCEROS (*DICEROS BICORNIS MICHAELI*).

Summary

There is a high incidence of irregular cyclicity in captive female black rhinos, and non-breeding females are less likely to express overt oestrous behaviours than proven breeders (Chapter 5). These two factors could play a role in the reduced reproductive output of the European captive population, and the high reproductive skew that exists between individuals (Chapter 4). However, the physiology underlying these anomalies is poorly understood. The aim of this chapter was therefore to investigate differences in reproductive (progesterone and oestradiol) and glucocorticoid metabolite concentration within females, to investigate whether any differences occur either between females, prior to, or during periods of regular and irregular cyclicity. Samples were collected every other day from both proven (N=6) and non-proven (N=12) females for between 9-15 months, so that differences in hormone concentrations within females could be investigated.

Faecal glucocorticoid metabolite (fGCM) concentration was significantly higher during periods of irregular cyclicity compared to during normal cycles, and furthermore, were higher during cycles longer than 40 days. However, when the data from proven and non-proven females were investigated separately, this effect was seen only in non-proven females. However, no differences in average fGCM were observed during the preceding luteal phase, follicular phase or luteal phase of the current cycle. This suggests that perhaps acute changes during the cycle rather than chronic changes in adrenal activity may be involved with extended oestrous cycle duration.

In both proven and non-proven females faecal oestradiol metabolite (fE_2) concentration was lower during periods of irregular cyclicity, and furthermore, fE_2 was significantly lower during acyclic periods and short oestrous cycles than during 20-40 day oestrous cycles. Although this could indicate potential differences in follicular development, overall fE_2 was highly variable both within and between females, and could not explain potential differences in the expression of oestrous behaviours between proven and non-proven females.

Faecal progesterone metabolite (fPG) concentrations were significantly higher during long cycles, indicating that as well as an extended luteal phase duration as is characteristic of this cycle type, fPG concentrations were also higher than during a normal 20-40 day cycle. However, average fPG concentration did not vary between cycle phases, or during the luteal phase prior to regular or irregular periods of cyclicity, or according to cycle type, and furthermore could not explain potential differences in the expression of oestrous behaviours between proven and non-proven females.

Although the average concentration of these three hormones did not tend to vary during the preceding luteal phases, the duration of this phase was significantly shorter than normal prior to both short oestrous cycles and acyclic periods. Furthermore, the preceding luteal phase was also significantly longer prior to longer cycles, indicating that the duration of prior hormone exposure may have some relevance on subsequent oestrous cycles exhibited.

In summary, differences in faecal hormone concentration have been observed between periods of regular and irregular cyclicity in captive black rhinos. Specifically, faecal glucocorticoids were higher during long cycle types and oestradiol lower during periods of acyclicity. Although the average concentration of progesterone, oestradiol or glucocorticoids during the preceding luteal phase did not predict cycle type, changes in the duration of this preceding luteal phase may be related to the different oestrous cycle types observed, and warrants further investigation.

7.1. Introduction

Among captive female black rhinoceros, both in Europe (Chapter 5) and in America (Brown et al. 2001), irregular oestrous cycles have been observed. In particular, four potential cycle types have been characterised during this study; short cycles lasting less than 20 days, normal cycles lasting between 20-40 days, long cycles lasting more than 40 days, and periods of acyclicity, where progesterone metabolites concentration remained at baseline for more than 10 days. Furthermore, results from the current study (Chapter 5) have also indicated that some of the irregular cyclicity observed in this population may be correlated with reproductive success. Specifically, longer cycles were seen more often in non-proven females, and among proven females, acyclic periods were seen more often in females that have not bred during the last seven years, compared to those that had bred more recently. Additionally, non-breeding females exhibit oestrus less often than breeding females, potentially limiting opportunities for successful introductions (Chapter 5).

Reduced reproductive output has been suggested as a major contributing factor to the limited growth and viability of *ex situ* rhinoceros populations (Chapter 4; Carlstead et al. (Carlstead et al. 1999a; Carlstead et al. 1999b); Smith and Read (Smith and Read 1992)), and reproductive skew could have profound consequences for maintenance of genetic diversity and the long-term viability of the captive breeding program. However, the hormonal basis of irregular cyclicity and inconsistent oestrous behaviours in the black rhinoceros are poorly understood, and a better understanding of the physiology underlying periods of regular and irregular cyclicity is required, to identify the potential causes and consequences of their occurrence.

One way in which oestrous cycles can be disrupted is through activation of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is activated in response to potential stressors, to allow the body to respond accordingly and maintain homeostasis (Moberg and Mench 2000). However, the presence of stressors can subsequently lead to the disruption of reproductive cyclicity and reduced fertility (Dobson et al. 2003; Dobson and Smith 2000). Research in domestic species, for example in cattle, sheep and pigs (Dobson et al. 2003; Dobson and Smith 2000; Turner et al. 2002; von Borell et al. 2007) have revealed some of the mechanisms by which stress-related disruption of reproduction may occur. Although the exact mechanisms are still under investigation, it is clear that disruption can occur on several different levels (Rivier and Rivest 1991), through alteration or inhibition of hormones from the hypothalamus, pituitary, and the

ovaries. Potential stressors may be physical or psychological in nature, and may be chronic (long-lasting), or acute (short-term); even acute stressors could lead to disruption if the timing is right, particularly during the pre-oestrous period, when a number of key hormonal changes take place. It is therefore important to understand whether any changes in adrenal activity may occur during the oestrous cycle, which could explain different cyclicity patterns observed in this population.

As well as exhibiting a higher proportion of extended cycles, non-proven female black rhinos in the EEP population were also less likely than proven females to exhibit regular oestrous behaviours (Chapter 5). Although this could in part be explained by the prevalence of irregular cyclicity within this population; in some female black rhinos, overt behavioural signs of oestrous may be absent when cyclicity patterns appear to be otherwise normal (Radcliffe et al. 2001). This issue has not been previously addressed in this species, and highlights the need to investigate hormone concentrations related to oestrous behaviour between females, and in relation to periods of regular and irregular cyclicity. Oestradiol from the developing follicle, in the relative absence of progesterone, is required for the expression of oestrous behaviours (Allrich 1994). Once a threshold concentration of oestradiol has been reached, oestrous behaviours will be expressed. However, this threshold may be different for different individuals, and could result in variation in the intensity of oestrous expressed.

The role of progesterone is also important for the expression of oestrous behaviour. Prior exposure may be required to facilitate the expression of oestrus behaviour in ewes (Fabre-Nys and Martin 1991a, b; Karsch et al. 1980), and has also been demonstrated to enhance the level of oestrus expression. Similarly, dairy cows with lower progesterone concentration prior to oestrus expressed behaviours with lower intensity (Walker et al. 2008). Furthermore, the phenomenon of silent oestrus has also been described in a number of species, which occurs when ovulation is not accompanied by oestrous behaviours, and is often associated with the first ovulation post-partum (Allrich 1994). The lack of overt oestrous behaviours is thought to be associated with an imbalance in progesterone and oestradiol. However, the exact mechanism regarding the influence of progesterone is not fully understood, and perhaps the duration of prior exposure could also be important. In the case of post-partum oestrous in cattle, high oestradiol in the final stages of pregnancy are thought to cause a refractory state in the brain, meaning it cannot respond to the normally oestrus-inducing concentration of oestradiol (Allrich 1994). Subsequent cycles are

generally accompanied by behaviours, as the progesterone produced by the corpus luteum re-sets the system and allows normal expression of oestrous behaviours. Additionally silent oestrus is also observed in seasonal breeders, often observed at the start and end of the breeding season, during the transition from ovulatory to anovulatory states (Asher et al. 2000; Herndon et al. 1987; Rivera et al. 2003).

This absence of overt oestrus behaviour has also been reported in a number of other endangered species, including Baird's tapir (Brown et al. 1994a), giant panda (Kersey et al. 2010), and Arabian leopards (van Dorsser et al. 2007); as well as in the black (Radcliffe et al. 2001), white (Hermes et al. 2006), and Sumatran (Roth 2006) rhinoceros. This has potentially serious implications for captive breeding programmes, where often animal managers must decide when to make introductions for breeding purposes. If behavioural signs of oestrous are not detected by human observers, introductions may not be made, or may be attempted at unsuitable times, reducing the chances of successful mating. It is therefore important to understand why differences in oestrous expression may occur in this population, and whether differences in oestradiol concentration may be related to irregular cyclicity, or whether prior exposure to either progesterone or oestradiol may be related to inconsistencies in behavioural expression both within and between females. Furthermore, as adrenal activity and the production of glucocorticoids can also inhibit the production of sex steroids, there is a possibility that glucocorticoids could also play a role in the inconsistent expression of oestrous behaviours.

Irregular oestrous cycles, and inconsistent expression of oestrus are two issues that have been highlighted in the European captive population of eastern black rhinoceros (Chapter 5). The aim of this chapter was to investigate reproductive cyclicity within females, to determine whether any differences in hormone metabolite concentration were apparent between different cycle types. Specifically, 1) to investigate whether any differences in reproductive or adrenal hormone metabolites were apparent between different cycle types, and 2) to determine whether differences in hormone secretion either during or prior to an oestrous cycle could explain the inconsistent behavioural signs of oestrus. Firstly, progesterone, oestradiol and corticosterone metabolite concentration were compared between regular and irregular periods of cyclicity, and between different cycle types (long, short, normal or acyclic). Secondly, to investigate whether the hormone status of an individual during the preceding oestrous cycle influences the type of cycle they subsequently exhibit, these hormones were also

compared during the luteal phase of the preceding cycle, the follicular phase of the current cycle during which follicle development occurs, and the luteal phase of the current cycle when progesterone is produced from the corpus luteum following ovulation. Thirdly, the duration of the preceding luteal phase was compared between cycle types, to determine whether the duration of prior hormone exposure influences the type of cycle an individual subsequently exhibits. Finally, any potential differences between proven and non-proven females were also considered in each of these cases.

7.2. Methods

7.2.1. Study population

This study included a subset of the females from Chapter 5, specifically 18 adult female black rhinoceros, between the ages of 5 and 28 years, housed at 8 institutions across Europe. The reproductive history of each individual was determined from the EAZA studbook, and of these females, 6 were categorised as proven, having previously produced at least one live calf, and 12 were categorised as non-proven, having never produced a live calf. As in Chapter 5, females recorded as only having had premature births were included in the 'non-proven' category.

7.2.2. Faecal sample collection and preparation

Faecal samples used for this study were collected from each female at least every other day for between 9 and 15 months. Samples were collected by keepers as soon as possible after defecation, taking multiple sub-sections from different areas of the faecal bolus to allow for potential uneven distribution within the sample, and combined in a zip-lock plastic bag. Samples were then frozen at -20°C following collection, and stored before shipment to Chester Zoo, UK for analysis.

Hormone metabolites were extracted from faecal samples according to an established wet-weight shaking extraction method (Edwards et al. 2013; Walker et al. 2002) (see Chapter 2 section 2.2.3 and Appendix 1 for detailed description of methods and protocols respectively). In brief, each sample was thawed, thoroughly mixed and weighed (0.5g±0.003g), before adding 5ml 90% methanol, vortexing and shaking overnight on an orbital shaker. Each sample was then vortexed and centrifuged for 20

minutes at 598g. The supernatant was decanted, dried under air, re-suspended in 1ml 100% methanol and the resulting faecal extract stored at -20°C until analysis.

7.2.3. Enzyme immunoassay

Previously described enzyme immunoassays adapted from Munro and Stabenfeldt, (1984), were used with some modifications to measure faecal progesterone (Walker et al. 2008), corticosterone (Watson et al. 2013) and oestradiol (Fanson et al. 2010) metabolites (see Chapter 2 section 2.2.4 and Appendix 1 for detailed description of methods and technique protocols respectively). Each EIA utilised an antiserum (monoclonal progesterone CL425, polyclonal corticosterone CJM006 or polyclonal oestradiol R4972; C.J. Munro, University of California, Davis); corresponding horseradish peroxidase (HRP) conjugated label (C.J. Munro, University of California, Davis); and standards (Sigma-Aldrich, UK) on a Nunc-Immuno Maxisorp (Thermo-Fisher Scientific, UK) microtitre plate. Black rhino faecal extracts were diluted as necessary in EIA buffer (1:70 for progesterone, 1:20 for corticosterone, and 1:30 for oestradiol), and run in duplicate (50µl for progesterone and corticosterone, 20µl for oestradiol) on the respective EIA's. Each faecal sample collected across the study period was analysed on between one and three EIAs. Samples collected at least every other day were analysed for progesterone metabolite concentration (N=2684), samples collected at least weekly were analysed for corticosterone metabolite concentration, plus additional samples were analysed around periods of regular and irregular cyclicity (N=2090), and samples collected at least every other day were also analysed for oestradiol metabolite concentration (N=2473).

7.2.4. Biochemical validation

EIAs were biochemically validated for measuring 1) progesterone, 2) glucocorticoid, and 3) oestradiol metabolites in female black rhino faecal extract through parallelism 1) R_2=0.969, $F_{1,7}$=222.140; 2) R_2=0.982, $F_{1,7}$=377.007; 3) R_2=0.986, $F_{1,7}$=506.114; all P<0.001) and matrix interference assessment 1) R_2=0.998, $F_{1,7}$=4338.484; 2) R_2=0.999, $F_{1,7}$=7133.701; 3) R^2=0.979 $F_{1,7}$=323.165; all P<0.001) (see Chapter 2, section 2.4 for full details). Intra- and inter-assay CVs for progesterone, corticosterone and oestradiol EIAs were 17.3%, 14.6% and 12.9%; 12.9%, 7.2% and 8.5%; and 9.3%, 8.3% and

16.8% for high and low binding synthetic and biological controls respectively. The cross reactivities for progesterone, corticosterone and oestradiol antisera have been reported elsewhere ((Walker et al. 2008), (Watson et al. 2013) and (Fanson et al. 2010) respectively) (See Appendix 2 for full details).

7.2.5. Data analysis

Oestrous cycles were determined from faecal progesterone metabolite concentration, and characterised according to a previously established method, where samples with baseline hormone concentrations are distinguished from those with elevated hormone concentrations, using an iterative process (Brown et al. 2001; Brown et al. 1994b). All non-pregnant samples from an individual female were used to calculate the mean and standard deviation (SD). An iterative process was then used to remove all samples greater than 1.5SD above the mean, before the mean was re-calculated and the process repeated until no samples exceeding 1.5SD from the mean remained. These samples were considered to have baseline concentrations of PG, and represented the follicular phase of the cycle. The onset of the luteal phase was considered to be the first sample where PG concentration exceeded 1.5SD above the mean, and the end of the luteal phase was considered to be when at least two consecutive samples were below the threshold of 1.5SD of the mean. As we were interested in any potential disruption that may occur during the follicular phase (period of follicle development prior to ovulation) and/or the subsequent luteal phase, a complete cycle was characterised as the first follicular phase sample to the last luteal phase sample, a slightly modified start and end criteria to that used in Chapter 5. Additionally the preceding cycle prior to the complete cycle or prior to any acyclic periods were also characterised with the same criteria.

Generalised linear mixed models (GLMM's) were used to investigate differences in faecal progesterone, oestrogen and glucocorticoid metabolite concentration as outlined below, using MLwiN version 2.02 (Rasbash et al. 2005). Normality tests were first conducted in IBM® SPSS® statistics version 20, and hormone data were $\log_{10}$ transformed to improve the distribution of data ($\log_{10}$ PG, $\log_{10}$ fGCM and $\log_{10}$ E_2).

Firstly, each sample collected and analysed on the respective EIAs was used to investigate differences in hormone concentration ($\log_{10}$ PG, $\log_{10}$ fGCM and $\log_{10}$ E_2)

across the different cycle types. To account for repeated sampling within and between females, random effects (date of sample collection and subject ID) were incorporated into the GLMM. Fixed effects of either cycle type (<20 days, 20-40 days, >40 days or acyclic) or cycle regularity (regular (20-40 day cycles) or irregular (<20 day, >40 day cycles and acyclic periods combined)) were added individually. As these were categorical GLMM, a reference category was assigned to each, using either 20-40 day cycles or regular periods of cyclicity as the reference category respectively, to which all other categories were compared. Comparisons were first made including samples from all females (N=18); GLMM were then repeated separating proven females (N=8) and non-proven females (N=12), to investigate whether differences existed according to prior reproductive success.

Secondly, average hormone concentrations (average $\log_{10}$ PG, average $\log_{10}$ fGCM and average $\log_{10}$ E_2) were used to investigate whether the occurrence of irregular cyclicity, or particular cycle types, may be related to the prior hormonal state of an individual. To investigate this, average hormone concentrations were calculated from faecal samples from 1) the luteal phase of the preceding cycle, 2) the follicular phase of the current cycle, and 3) the luteal phase of the current cycle. As multiple oestrous cycles were used from each female, cycle number (1-17) and subject ID were incorporated into the model as random effects. Again, fixed effects of either cycle type or cycle regularity were added individually, and reference categories assigned as previously described. Comparisons were first made including samples from all females (N=18), and then repeated separating proven females (N=8) and non-proven females (N=12), to investigate whether differences existed according to prior reproductive success, and differences between reproductive categories are shown where relevant.

Finally, the potential influence of duration of prior hormone exposure was investigated, using the length (days) of the preceding luteal phase as the dependent variable, and comparing by cycle regularity and cycle type as described above. All GLMMs utilised a normal error structure, and the significance of each fixed effect was determined using the Wald statistic and chi-squared (χ^2) distribution, with alpha set to 0.05.

7.3. Results

7.3.1. Oestrous cycles

A total of 175 oestrous cycles were characterised across the study period; 124 cycles in non-proven females and 51 cycles in proven females. Of these cycles, 45 were less than 20 days in length (<20d), 99 were between 20 and 40 days (20-40d), and 31 were longer than 40 days (>40d) (Figure 7.1). Additionally, 24 acyclic periods were observed, occurring in 12 (5 proven and 7 non-proven) out of 18 females, and lasting between 14 and 133 days. All 18 females exhibited periods of both regular (20-40d) and irregular (<20d, >40d or acyclic) cyclicity during the sampling period. For all further analyses, these 199 periods of potential oestrous cyclicity were used.

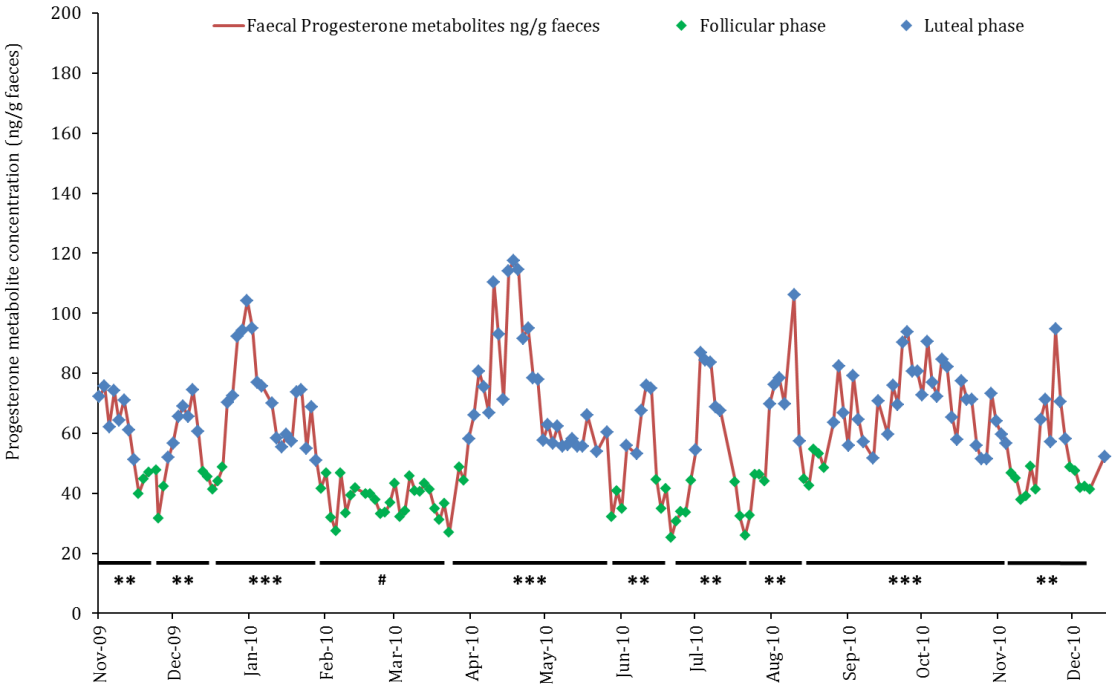


Figure 7.1: Individual progesterone profile of oestrous cycles in a female black rhino, representing follicular and luteal concentration samples as determined using the iteration method. Cycle lengths are represented by solid lines, and are categorised as short (*), normal (**), long (***) or acyclic periods (#).

7.3.2. Progesterone and irregular cyclicity

When comparing log_{10} PG within females according to cycle regularity, log_{10}PG were higher during periods of irregular cyclicity compared to periods of regular cyclicity (χ^2=8.775, df=1, P=0.003). Although there were no differences in log_{10}PG between short cycles and normal cycles of 20-40 days (χ^2=0.026, df=1, P=0.87), differences were observed between other cycle types. Log_{10} PG was significantly higher during long cycles than during normal cycles (χ^2=145.279, df=1, P<0.001), or during short cycles (χ^2=84.313, df=1, P<0.001). Additionally, log_{10}PG was significantly lower during acyclic periods than during normal cycles (χ^2=73.337, df=1, P<0.001), short cycles (χ^2=44.746, df=1, P<0.001), or long cycles (χ^2=319.390, df=1, P<0.001). Although there is some circularity in these analyses, as acyclic periods are characterised by a sustained (more than 10 days; (Brown et al. 2001)) concentration at baseline, the difference observed between normal and long cycles may have some biological relevance beyond this. Long cycles are characterised as cycles longer than 40 days in length, but these results indicate that not only is progesterone concentration elevated above baseline for a longer period, but is also of a higher concentration than during a normal 20-40 day cycle.

These differences in log_{10}PG concentration between cycle types were consistent in both proven and non-proven females. In both proven (χ^2=2.240, df=1, P=0.13) and non-proven χ^2=0.794, df=1, P=0.37 females, there was no difference in log_{10}PG between normal and short cycles. However, log_{10}PG concentration was significantly lower during acyclic periods in both proven (χ^2=41.057, df=1, P<0.001) and non-proven (χ^2=33.783, df=1, P<0.001) females, and was significantly higher during long cycles in both proven (χ^2=24.893, df=1, P<0.001) and non-proven (χ^2=125.342, df=1, P<0.001) females. However in proven females, there was no overall difference in log_{10}PG between regular and irregular periods of cyclicity (χ^2=1.674, df=1, P=0.20), although this difference was still apparent in non-proven females (χ^2=220.610, df=1, P<0.001).

The average log_{10}PG concentration during the previous luteal phase was no different prior to regular and irregular periods of cyclicity (χ^2=0.446, df=1, P=0.50), or prior to long (χ^2=0.288, df=1, P=0.59), short (χ^2=0.532, df=1, P=0.47) or acyclic periods (χ^2=0.002, df=1, P=0.96) as compared to normal cycles. This indicates that the progesterone concentration during the preceding luteal phase does not appear to influence the type of cycle that will be exhibited next. Progesterone concentration should be at baseline during the follicular phase of the oestrous cycle (Figure 7.1),

however, the average log_{10}PG concentration during the follicular phase of the cycle tended to be higher during irregular periods of cyclicity (χ^2=3.798, df=1, P=0.051) than during regular periods. Comparisons between cycle types revealed that this was predominantly driven by higher concentrations during acyclic periods (χ^2=6.241, df=1, P=0.012), as both short (χ^2=0.357, df=1, P=0.55) and long (χ^2=2.277, df=1, P=0.0.14) cycles were no different to normal cycles. This indicates that although acyclic periods are characterised by progesterone metabolite concentration that does not increase above baseline, there does appear to be a higher concentration than during a typical follicular phase, but not sufficient to be indicative of luteal activity.

Although when all faecal samples were compared log_{10} PG was significantly higher during long cycles than during normal or short cycles (above), when comparing the average concentration during the luteal phase of the oestrous cycle, this observation was not quite as apparent. There was a tendency for average log_{10}PG concentration to be higher during long cycles in non-proven females (χ^2=2.764, df=1, P=0.096), but this was not consistent in proven females (χ^2=0.006, df=1, P=0.94).

There were no consistent differences in average log_{10} PG between proven and non-proven breeders, either in the preceding luteal phase (χ^2=0.197, df=1, P=0.66), follicular phase (χ^2=0.056, df=1, P=0.81), or the current luteal phase (χ^2=0.377, df=1, P=0.54). Therefore, using this approach, differences in prior exposure to progesterone does not explain why non-proven females are less likely to exhibit overt behavioural signs of oestrus.

7.3.3. Oestrogens and irregular cyclicity

Faecal oestrogen metabolites were highly variable both within and between females (Figure 7.2), ranging from around 20 to 400 ng/g faeces. Although there were some clear peaks observed during the follicular phase, there were other peaks observed during the luteal phase, making it difficult to decipher follicular activity (Figure 7.2). A number of females exhibited sustained low concentration that lasted from 6 to 23 weeks. In 8 females, this low concentration occurred between October and December, indicating that this could indicate some seasonal differences in oestradiol concentration; however, cyclicity based on progesterone profiles was unaffected during this time (Figure 7.2).

Controlling for repeated sampling within multiple females, there was no overall difference in faecal oestradiol metabolite concentration ($\log_{10} E_2$) between proven and non-proven females (χ^2=0.519, df=1, P=0.47). However, when comparing $\log_{10} E_2$ within females according to cycle regularity, $\log_{10}E_2$ was lower during periods of irregular cyclicity (<20d and >40d cycles and acyclic periods combined) compared to periods of regular cyclicity (20-40d cycles) (χ^2=6.269, df=1, P=0.012). Furthermore, when comparing all four cycle types, $\log_{10} E_2$ was lower during periods of acyclicity than during 20-40d cycles (χ^2=38.394, df=1, P<0.001), short cycles (<20d) (χ^2=13.897, df=1, P<0.001) and long cycles (>40d) (χ^2=42.466, df=1, P<0.001). Additionally, $\log_{10} E_2$ was also lower during short cycles, as compared to long cycles (χ^2=4.055, df=1, P=0.044). In both of these cases, lower oestradiol metabolite concentration could be related to a reduction in follicular activity, either associated with acyclic periods where perhaps pre-ovulatory follicles do not develop sufficiently to ovulate, and hence no progesterone-secreting corpus luteum is formed, or in the case of short cycles, perhaps the follicles that do develop may be smaller, producing a lower concentration of oestradiol.

When data were separated by reproductive category, this difference in $\log_{10} E_2$ was apparent in both proven and non-proven females. In proven females, $\log_{10} E_2$ was significantly lower during periods of acyclicity compared to normal cycles (χ^2=13.196, df=1, P<0.001), short cycles (χ^2=5.209, df=1, P=0.022) and long cycles (χ^2=13.683, df=1, P<0.001). Similarly, in non-proven females, $\log_{10} E_2$ was significantly lower during periods of acyclicity compared to normal cycles (χ^2=25.654, df=1, P<0.001), short cycles (χ^2=8.067, df=1, P=0.005) and long cycles (χ^2=28.191, df=1, P<0.001). Overall, $\log_{10} E_2$ tended to be lower during periods of irregular cyclicity compared to regular cyclicity, as the relationship was close to significance in both proven (χ^2=3.495, df=1, P=0.062) and non-proven females (χ^2=3.805, df=1, P=0.051).

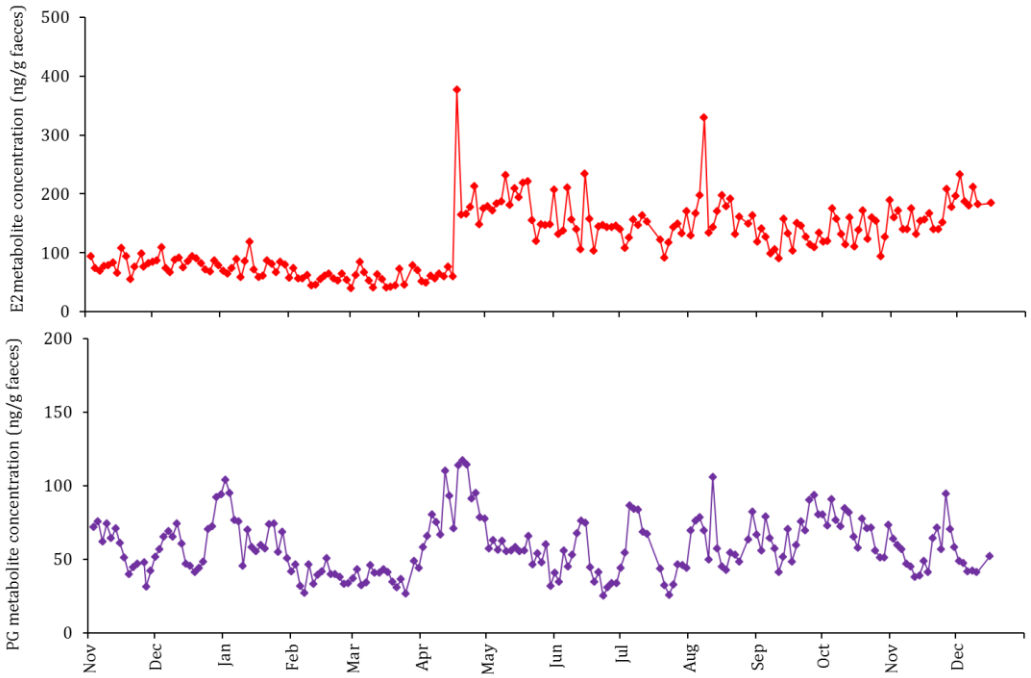
There were no differences in average $\log_{10} E_2$ concentration between periods of regular and irregular cyclicity, either during the preceding luteal phase (χ^2=0.199, df=1, P=0.66) or during the follicular phase of the current cycle (χ^2=1.301, df=1, P=0.25). Similarly, there were no differences in average $\log_{10} E_2$ concentration during the preceding luteal phase between cycle types, with short (χ^2=0.044, df=1, P=0.83), long (χ^2=0.960, df=1, P=0.33) and acyclic periods (χ^2=0.043, df=1, P=0.84) no different from normal cycles. The same was true for the follicular phase, with no differences between cycle types, with short (χ^2=0.037, df=1, P=0.85), long (χ^2=2.441, df=1, P=0.12) and

acyclic periods (χ^2=0.669, df=1, P=0.41) no different from normal cycles. Again, the prior exposure to oestradiol does not appear to influence subsequent oestrous cycle type.

However, during the current luteal phase, average $\log_{10}$ E_2 concentration tended to be lower during irregular periods of cyclicity compared to regular periods (χ^2=2.815, df=1, P=0.09), with average $\log_{10}$ E_2 concentration significantly lower during short cycles as opposed to 20-40 day cycles (χ^2=0.5.395, df=1, P=0.02).When separated by reproductive category, this was true for non-proven females (χ^2=0.3.892, df=1, P=0.049), but there were no differences between short and normal cycles in proven females (χ^2=1.354, df=1, P=0.24).

There were no consistent differences in average $\log_{10}E_2$ between proven and non-proven breeders, either in the preceding luteal phase (χ^2=0.325, df=1, P=0.57), follicular phase (χ^2=0.413, df=1, P=0.52), or the current luteal phase (χ^2=0.165, df=1, P=0.68). As there were no overall differences in prior exposure to oestradiol between proven and non-proven females, this also does not appear to explain why non-proven females are less likely to exhibit overt behavioural signs of oestrus.

(a)



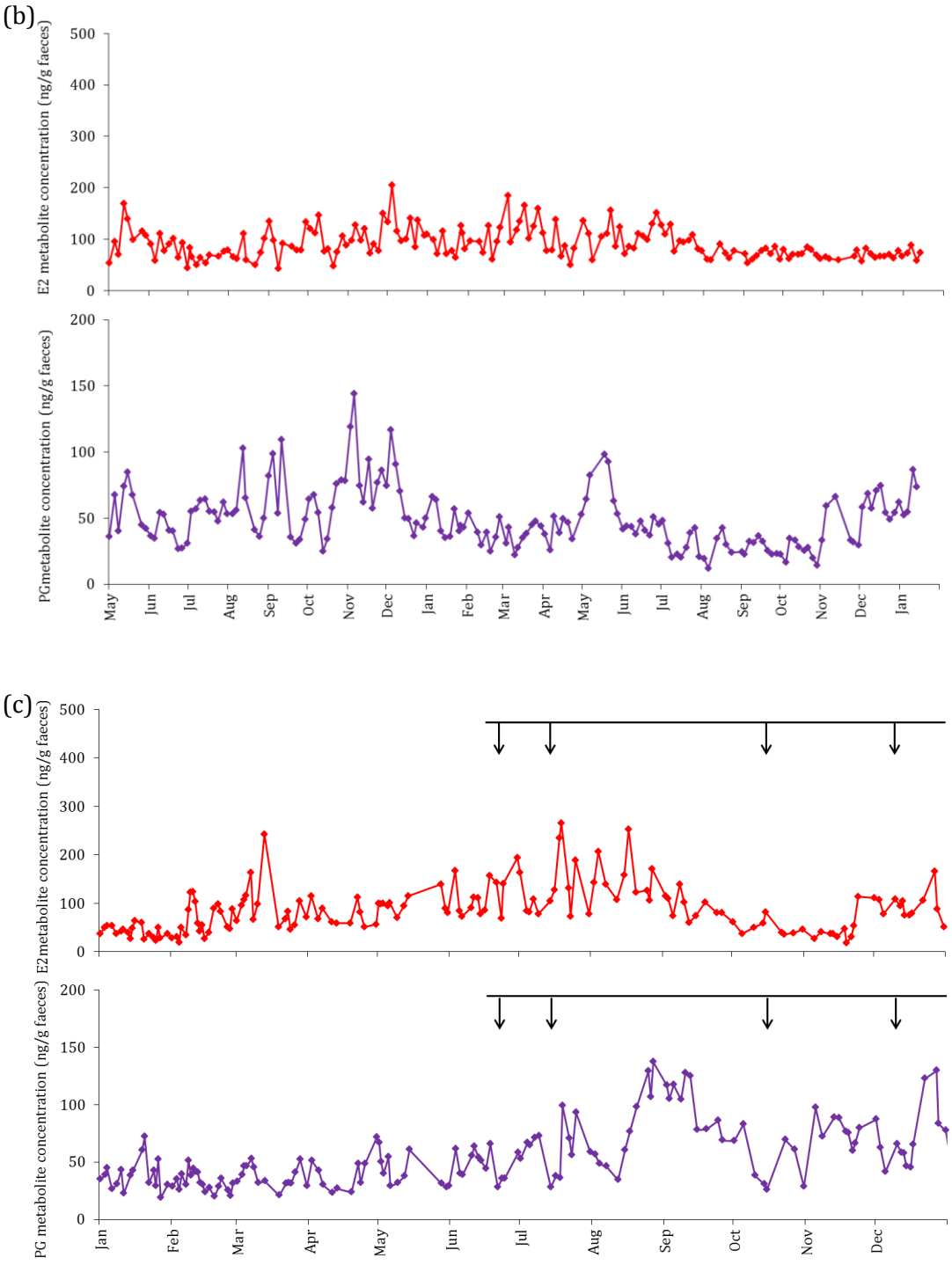


Figure 7.2: Individual profiles of faecal oestradiol metabolite (E_2; red) and faecal progesterone metabolite (PG; purple) concentration. Arrows on figure (c) represent when mating occurred.

7.3.4. Glucocorticoids and irregular cyclicity

When comparing $\log_{10}$ fGCM within females according to cycle regularity, $\log_{10}$ fGCM were higher during periods of irregular cyclicity compared to periods of regular cyclicity (χ^2=5.052, df=1, P=0.025). Furthermore, when comparing all four cycle types, $\log_{10}$ fGCM was higher in long cycles (>40d) than during 20-40d cycles (χ^2=16.905, df=1, P<0.001), short cycles (<20d) (χ^2=5.461, df=1, P=0.019) and periods of acyclicity (χ^2=17.505, df=1, P<0.001) (Figure7.3).

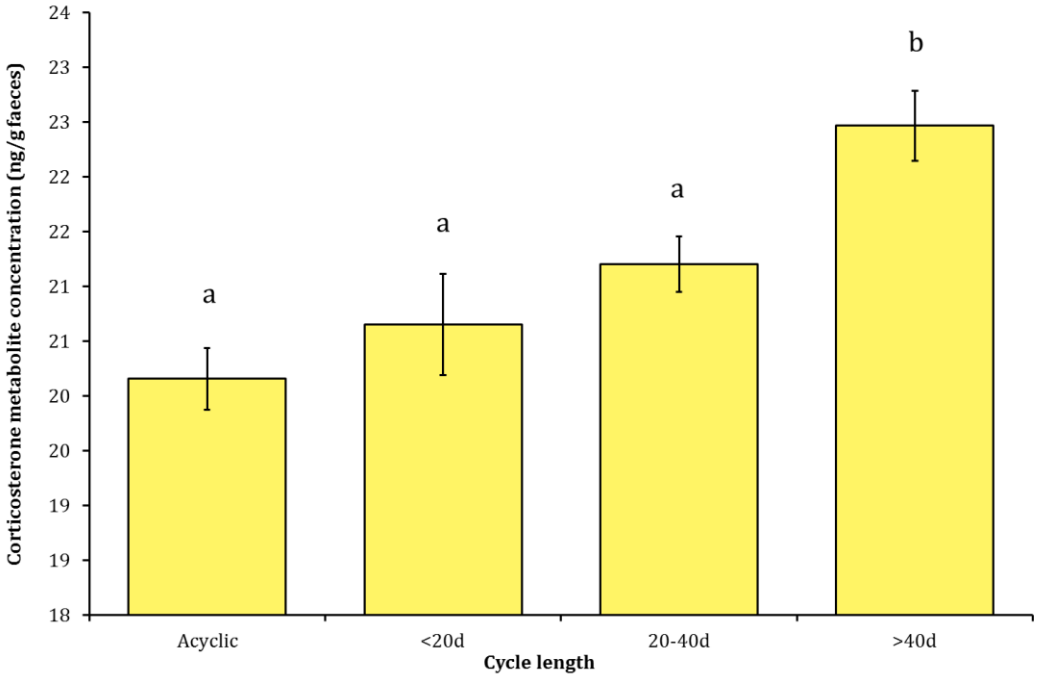


Figure 7.3: Faecal glucocorticoid metabolite (fGCM) concentration during normal, short and long cycles, and acyclic periods. Bars represent mean fGCM+/-1SE; letters (a, b) reflect significant differences.

However, when data were separated by reproductive category, this difference in $\log_{10}$ fGCM was only apparent in non-proven females, with higher $\log_{10}$ fGCM during periods of irregular cyclicity (χ^2=4.750, df=1, P=0.029), and specifically higher $\log_{10}$ fGCM concentration during long cycles (>40d) than during 20-40d cycles (χ^2=20.285, df=1, P<0.001), short cycles (<20d) (χ^2=6.796, df=1, P=0.009) and periods of acyclicity (χ^2=24.960, df=1, P<0.001). In proven females, no differences in $\log_{10}$ fGCM were

observed either between periods of regular and irregular cyclicity (χ^2=0.235, df=1, P=0.63), or between cycle types (all P>0.05).

There were no differences in average $\log_{10}$ fGCM during the preceding luteal phase (χ^2=0.969, df=1, P=0.32) prior to either regular or irregular periods of cyclicity. Similarly, there were no differences according to cycle type, with average $\log_{10}$ fGCM no different during the luteal phase prior to long (χ^2=1.044, df=1, P=0.31), short (χ^2=0.282, df=1, P=0.60) or acyclic periods (χ^2=0.227, df=1, P=0.63), compared to before normal cycles. This indicates that fGCM concentration in the preceding period does not predict whether a normal or irregular cycle type may occur.

Although elevated $\log_{10}$ fGCM was observed during long cycles, when controlling for repeated sampling within individuals, using average $\log_{10}$ fGCM to compare between the follicular and luteal phase of different cycle types did not show this effect. There were no differences between regular and irregular periods of cyclicity, either during the follicular phase (χ^2=0.561, df=1, P=0.45), or the luteal phase (χ^2=0.442, df=1, P=0.51) of the current cycle. Furthermore, there were no differences in average $\log_{10}$ fGCM during the follicular phase of the current cycle between long (χ^2=0.001, df=1, P=0.97), short (χ^2=0.484, df=1, P=0.49), or acyclic periods (χ^2=1.028, df=1, P=0.31), compared to normal cycles, indicating that changes in average $\log_{10}$ fGCM during the follicular phase do not predict the subsequent occurrence of irregular cycle types. Similarly, there were no differences in average $\log_{10}$ fGCM during the luteal phase of long (χ^2=0.001, df=1, P=0.97) or short (χ^2=0.906, df=1, P=0.34) cycles, compared to normal cycles between 20-40 days in length. Although this is not consistent with the previous finding above, this may reflect the decreased sensitivity of using average concentrations, when changes in hormone concentration are relatively small.

7.3.5. Duration of prior hormone exposure

The length of the preceding luteal phase was compared between cycle types, to determine whether the duration, as opposed to the concentration of prior hormone exposure may influence the type of cycle that subsequently occurs. In all females, both proven and non-proven alike, the length of the preceding luteal phase varied between regular and irregular periods of cyclicity (χ^2=4.781, df=1, P=0.029). when broken down by cycle type, the luteal phase prior to long cycles were significantly longer than the

luteal phase prior to 20-40 day cycles (χ^2=294.836, df=1, P<0.001). Additionally, the length of the preceding luteal phase was significantly shorter prior to short cycles (χ^2=12.163, df=1, P<0.001), and acyclic periods (χ^2=26.901, df=1, P<0.001), compared to those cycles of 20-40 days in length, and the previous luteal phase was also shorter prior to acyclic periods compared to short cycles (χ^2=4.911, df=1, P=0.027). This indicates that although there was no clear evidence that the average concentration of progesterone, oestradiol or glucocorticoids during the preceding cycle influenced the subsequent cycle type, perhaps the duration of hormone exposure during the preceding luteal phase may be related to the type of cycle that occurs subsequently.

7.4. Discussion

All of the females included in this study exhibited periods of both regular (cycles 20-40 days in length), and irregular (cycles <20 day or >40 days in length, or periods of acyclicity) oestrous cyclicity over the course of a year. We have previously demonstrated (Chapter 5) that the longer cycle types are more often observed in non-proven females, and among proven females, acyclic periods are more often seen in females that have not bred for at least seven years. Furthermore, results from Chapter 5 have also indicated that non-breeding females are also less likely to exhibit behavioural signs of oestrus on a regular basis. As these two factors may have potential consequences for reproductive success, it is important to determine what may be causing these periods of irregular cyclicity, and why differences in oestrus expression may occur. In this chapter, we have investigated the influence of previous and current hormone exposure on cycle regularity and cycle type.

The oestrous cycle is controlled by a delicate balance of hormones from the hypothalamic-pituitary-ovarian axis, which can be prone to disruption, particularly during the period preceding oestrus (Dobson and Smith 2000). One area of investigation for this study was the role of adrenal activity, which can interfere with normal reproductive function at a number of levels along the hypothalamic-pituitary-ovarian axis (Wingfield and Sapolsky 2003). Indeed, a key finding of this study was that the occurrence of long oestrous cycles was accompanied by an increased concentration of faecal glucocorticoids, compared to any other cycle type, particularly in non-proven females. One potential mechanism by which adrenal activity can disrupt reproduction is through alteration of gonadotropin-releasing hormone (GnRH), and subsequently

luteinising hormone (LH) pulsatility (Dobson and Smith 2000), either at the level of the hypothalamus, pituitary, or both. Timing of these pulses is crucial for normal follicular development and ovulation, so if the correct sequence of events does not occur, there is the potential for disruption. One possible scenario is that GnRH/LH pulse frequency is sufficient for follicular growth, but insufficient to result in the LH surge required for ovulation; resulting in an anovulatory follicle (Dobson and Smith 2000). The presence of anovulatory follicles has been reported in a number of species (Lopez-Gatius et al. 2001; McCue and Squires 2002; Veiga-Lopez et al. 2006; Wiltbank et al. 2002), including the black rhinoceros (Radcliffe et al. 2001). Ultrasound investigation of ovarian events in two black rhino females revealed three incidences of such structures which formed at the time of expected ovulation, but were not associated with signs of follicular collapse or formation of a corpus luteum. Corresponding hormone analysis in one such case revealed an erratic progesterone profile during this time, although glucocorticoids were not investigated. Although these events were all observed during October/November and were considered to be a seasonal occurrence, we have observed erratic patterns of cyclicity and long oestrous cycles across all months of the year (Chapter 5), indicating that irregular cyclicity is not restricted to seasonal differences. The data presented here indicate that increased adrenal activity could be related to extended oestrous cycles in females black rhinoceros.

As indicated in Chapter 5, clear differences in the expression of overt oestrous behaviours occur between breeding and non-breeding females, and a better understanding of what may be limiting the expression of overt behaviours would benefit management of this captive breeding programme, and the species in general. However, similar to that reported by Brown et al. (2001) for captive black rhinoceros in America, faecal oestradiol metabolite concentration was highly variable both within and between females included in this study. Some clear peaks of oestradiol metabolite concentration occurred during the follicular phase, which might be associated with follicle development and ovulation, but other increases in concentration were also observed during the luteal phase, making it difficult to decipher follicular activity. However, average $\log_{10}$ E_2 concentration was lower during periods of acyclicity, and during short cycles, when compared to normal cycles of 20-40 days. The lower concentration of oestradiol metabolites during acyclic periods indicates that there may be reduced follicular development. This could result in the lack of a pre-ovulatory follicle, and may be the reason that no corpus luteum appears to form during these periods to secrete the progesterone reflected by the cyclic pattern in a normal oestrous

cycle. Similarly, short cycles may be associated with lower oestradiol than normal cycles, as although follicles must develop and lead to ovulation in order to see the cyclic pattern in progesterone metabolites, they may perhaps be smaller, and therefore result in a lower concentration of oestradiol metabolites being secreted. However, there do not appear to be any clear differences in faecal oestradiol concentration between proven and non-proven females that could explain the differences in expression of oestrus.

Oestradiol, in the relative absence of progesterone prior to ovulation acts upon the hypothalamus to induce oestrus (Allrich 1994). Although oestradiol is considered to be the primary hormone driving expression of oestrous behaviours, the relative concentration of both progesterone and glucocorticoids were also investigated here. The prior exposure of progesterone may also be important to ensure behaviours are expressed (Walker et al. 2008). Additionally increased adrenal activity, and the subsequent release of glucocorticoids could interfere with the production of both oestradiol and progesterone (Wingfield and Sapolsky 2003), potentially disrupting the normal expression of oestrous behaviours. However, in this study there were no clear differences observed between regular and irregular periods of cyclicity, or in overall hormone concentrations between breeding and non-breeding females that might help to explain the inconsistent expression of oestrous behaviours.

However, it is possible that using faecal samples may not be the best sample medium to investigate oestrogens in the black rhinoceros. Although the route of excretion of different steroids has not been conducted in this species, in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) (Heistermann et al. 1998)and African elephant (*Loxodonta africana*)(Wasser et al. 1996), progesterone metabolites are mainly excreted into the faeces, whereas oestrogen metabolites are primarily excreted via the urine (Hodges et al. 2010). It may therefore be the case that oestradiol metabolites in faeces only represent a small portion of the biologically active hormone present in the circulation, perhaps supported by the low response in faecal oestradiol metabolites observed following GnRH vaccination (Chapter 2). An alternative approach, such as using urine, may be required to investigate biologically relevant changes in oestradiol concentration that may help to understand differences in expression of oestrus.

Secondly, the use of average hormone concentrations to investigate differences during the preceding oestrous cycle phase may not allow sufficient detail into changes that may occur during this period. Differences in hormone concentration often appear to be

quite subtle, so the benefit of longitudinal sampling is that these differences can be detected over time. However, the use of average hormone concentration is commonly used in this field, particularly when faecal samples are collected opportunistically. This may not always be the right approach when investigating short-term changes in hormone concentration (Edwards et al. 2013); Hill et al. *in prep*; Trotter et al. *in prep.*), as average hormone concentration may dampen the variation in hormone concentration that is of interest. This therefore highlights the importance of looking at changes in hormone concentration within individuals over time. Perhaps a longitudinal investigation of oestrogens, using urine samples alongside faeces, and detailed records of when oestrus behaviours are exhibited may prove beneficial.

The duration of the preceding luteal phase did appear to vary between the different cycle types, indicating that the prior hormone state of an individual may indeed be of relevance to their subsequent cyclicity. The preceding luteal phase was significantly shorter prior to short cycles and shorter still prior to acyclic periods. One potential similarity between short and acyclic periods, is that perhaps follicular development is sub-optimal, supported by the lower oestradiol concentration also observed during these two cycle types. Perhaps a shorter preceding luteal phase, and corresponding shorter duration of exposure to progesterone, may have a negative effect on follicular development. This has been observed in humans, where women that have menstrual cycles with a short luteal phase exhibit poor follicular development (Sherman and Korenman 1974; Smith et al. 1985; Strott et al. 1970). This could result in smaller follicles that either fail to ovulate, in the case of acyclic periods, or ovulate but may have a weaker corpus luteum, resulting in shorter oestrous cycle length. The preceding luteal phase was also significantly longer prior to long cycles, which could also alter follicular development, resulting in prolonged growth (Fortune et al. 1991). Although further investigation is required into this relationship between prior hormone exposure and subsequent oestrous cycle length, this finding does indicate that even subtle changes in the hormone milieu could impact subsequent cyclicity.

In summary, differences in faecal hormone concentration have been observed between periods of regular and irregular cyclicity in captive black rhinos. Specifically, faecal glucocorticoids were higher during long cycle types and faecal oestradiol metabolites lower during periods of acyclicity. However, oestradiol, progesterone or glucocorticoid concentration during the oestrous cycle, or the preceding luteal phase could not explain potential differences in the expression of oestrous behaviours. However, the

duration of the preceding luteal phase, was related to oestrous cycle types, highlighting that perhaps the prior hormone exposure may indeed be of relevance to the occurrence of a normal oestrous cycle, perhaps via the effect on follicular development, and therefore warrants further investigation.

7.5. Conclusion

- As previously demonstrated in Chapter 5, oestrous cycles of varying length are apparent in female black rhinoceros, including cycles shorter (<20 days) and longer (>40 days) than the normal cycle length of 20-40 days; periods of acyclicity are also observed.
- Furthermore, inconsistent expression of oestrus may be related to differential reproductive success (Chapter 5).
- Changes in hormone concentration during the current and preceding oestrous cycle were investigated, to determine whether differences in the hormone milieu influence 1) the subsequent occurrence of these different cycle types, or 2) the differential expression of oestrous behaviour between proven and non-proven females.
- Faecal glucocorticoid metabolite (fGCM) concentration was higher during periods of irregular cyclicity compared to during normal cycles, and furthermore, were higher during longer cycles. This relationship was observed in non-proven females, but not in proven females.
- Faecal oestradiol metabolite (fE_2) concentration was lower during periods of irregular cyclicity, and furthermore, in both proven and non-proven females, fE_2 was significantly lower during acyclic periods, and during shorter cycles (<20 days) than during normal oestrous cycles (20-40d).
- However, there were no consistent differences in average oestradiol concentration between proven and non-proven females that could explain the differences in behavioural oestrus.
- Furthermore, there were no differences between the progesterone, oestradiol or glucocorticoid metabolite concentrations observed during the luteal phase prior to regular or irregular cycles.
- Similarly, no differences were observed during the follicular or luteal phases of the different cycle types that explain their occurrence.

- However, the duration of the preceding luteal phase was significantly longer prior to a long cycle, and significantly shorter prior to both short cycles and acyclic periods. The duration of the preceding luteal phase may influence follicular development, and in this way could influence the type of oestrous cycle observed.

7.6. Acknowledgements

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- Zoo Dvůr Králové, Dvůr Králové, Czech Republic
- Ree Park Ebeltoft Safari, Ebeltoft, Denmark
- Zoo Hannover, Hannover, Germany
- Zoo Krefeld, Krefeld, Germany
- Zoologischer Garten Köln, Köln, Germany
- Paignton Zoo, Paignton, UK
- Port Lympne Wild Animal Park, Hythe, Kent, UK
- Howletts Wild Animal Park, Bekesbourne, Kent, UK
- Zoo Zürich, Zurich, Switzerland
- Zoologischer Garten Magdeburg, Magdeburg, Germany
- Zoo de Pont-Scorff, Pont-Scorff, France

CHAPTER 8

8. GENERAL DISCUSSION AND OVERALL CONCLUSIONS

The global extinction crisis means that each day more species move closer to extinction, as habitat is lost and species are placed under increasing pressure due to anthropogenic causes (Magurran and Dornelas 2010). *Ex situ* conservation and captive breeding programmes have an important role to play, providing a reservoir to protect against further species decline, whilst also raising public awareness of global conservation issues, and financial support for *in situ* conservation. Furthermore, maintaining healthy *ex situ* populations allows a controlled environment in which to learn about the biology of species that may be difficult to observe in the wild, providing scientific knowledge to better coordinate global conservation efforts.

For *ex situ* populations to fulfil these important roles, they must be self-sustaining, both demographically and genetically to ensure that they can act as a suitable reserve for their *in situ* counterparts. This means they must be self-replacing often without any further supplementation from the wild (Leus et al. 2011b), and maintain sufficient genetic diversity representative of their wild counterparts, so as not to lose the potential for natural behaviour and future adaptation (McPhee and Carlstead 2010). There is increasing pressure for captive breeding programs to adopt an evidence-based approach to ensure that populations are meeting increasingly strict criteria for population viability (Lacy 2013). However, more recently, it has been suggested that many cooperative breeding programs are failing to reach these targets (Conway 2011; Leus et al. 2011a; Long et al. 2011), and these initial criteria may not be sufficiently strict to preserve the viability of *ex situ* populations in the longer term (Lacy 2013).

With fewer than 5000 black rhinoceros (*Diceros bicornis*) in the wild and the on-going threat of poaching jeopardising the survival of remaining populations, *ex situ* breeding programmes play an important role for the conservation of this critically endangered species. The eastern subspecies (*D. b. michaeli*) is currently the most threatened of the three remaining subspecies, with only around 800 individuals remaining in Kenya, Tanzania and an out of range population in South Africa. Coordinated captive breeding programs have the potential not only to act as an insurance policy against further decline, but also provide the potential for reintroduction should conditions allow. However, *ex situ* populations are currently under-performing, with inconsistent rates of reproduction one of the factors limiting population growth. The aim of this thesis was to gain a better understanding of factors that influence population performance and reproductive success in the European captive population of eastern black

rhinoceros, in an attempt to improve population performance and maximise the conservation potential of the breeding program for this species.

- *The first aim of this thesis was to gain a better understanding of factors that influence population performance in this species, to determine biological limits for key demographic parameters relating to fecundity, mortality and population structure, and identify where there may be potential to improve population performance.*

In Chapter 3, demographic information on eight populations of eastern black rhinoceros within managed reserves, provided by Kenya Wildlife Service (KWS), were used to calculate a number of key demographic parameters. Firstly, historical data and simulated population projection models were used to determine the current performance of *in situ* populations, and how population growth rates vary under natural conditions. Although projected population growth rates were quite variable between reserves, ranging from 2.26 to 7.04% per annum, six of the eight reserves were projected to exceed a target growth rate of at least 5% per annum.

Secondly, demographic data from the last 25 years were used to estimate several indicators of population performance relating to mortality, reproduction and population structure, to establish both the normal range and variability between individuals and across reserves. These parameters were compared to previously established targets which were designated as the minimum necessary to attain 5% growth per annum. In general, measures of population structure and mortality were achieving optimal targets; however, even in reserves exceeding 5% growth per annum, average reproductive parameters were not always being achieved. A high degree of variation was observed between individuals, particularly in age at first reproduction and inter-birth interval, and at a population level, the percentage of females breeding each year. This highlights that even though most of these populations are growing at an acceptable rate, there are still improvements that could be made to maximise performance. In particular, individual monitoring to determine individuals that are not meeting the necessary targets would be beneficial, to identify individuals or sub-populations that may require additional management to improve their success, and ensure that all individuals have the potential to contribute genetics to future generations.

Demographic monitoring of *in situ* populations of black rhinoceros not only provides useful information to guide biological management of these reserves, but can also be used as a reference for the *ex situ* population, to determine where there may be room for improvement. In Chapter 4 of this thesis, demographic information from the EEP studbook for the eastern black rhinoceros was used to perform population viability analysis to determine whether the European captive population is currently self-sustaining. Furthermore, differences in demographic parameters were compared within this population over time, and between *in situ* and *ex situ* populations of eastern black rhinoceros, to identify factors that may be limiting population performance *ex situ*.

Although the EEP population is demographically self-sustaining, it is currently only growing at around 1-2% per annum, well below the desired target of 5%. Data from the last 10 years has revealed a reduction in fecundity compared to the last 25 years, with a lower percentage of adult females breeding each year. Furthermore, only 37.1% of males and 36.1% females from the European studbook of *D. b. michaeli* have produced offspring in their lifetime. This historical reproductive skew is also reflected in the current population, with 42.1% of mature males and 48.6% of mature females yet to reproduce, with a further 16.7% and 39.1% of previously proven males and females that have not bred for over seven years. Compared to the *in situ* reference populations, females begin breeding later, have longer inter-birth intervals, and a lower proportion of females reproduce each year. Overall, the high proportion of non-breeding males and females within this population could be the main factor limiting the performance of this population.

- *As sub-optimal reproduction is the main factor limiting the performance of the EEP population, both in terms of total reproductive output, and unequal contribution between individuals, the next aim of this thesis was to investigate intrinsic differences between breeding and non-breeding males and females within the population.*

In Chapter 5, faecal hormone analysis was conducted on approximately 90% of the EEP population of eastern black rhinoceros, to establish the current reproductive status of both males and females within this population. The purpose of this was to characterise oestrous cyclicity based on faecal progesterone metabolite concentration, and determine how regularly females were cycling, and to establish testosterone

concentration and variability among males. Furthermore, comparisons were made between breeding and non-breeding individuals to determine whether intrinsic differences in reproductive hormones may be related to reproductive success.

Although all females exhibited at least some evidence of cyclicity, a high incidence of irregular cyclicity was also observed. Similar to other studies, typical cycles were considered to be 20-40 days in length, but cycles both shorter (<20 days) and longer (>40 days) than normal were observed, as were periods of acyclicity, where faecal progesterone metabolite concentration remained at baseline for periods up to 17 weeks. Although all cycle types were observed in both proven and non-proven females, long cycles were exhibited more often in non-proven females, while among proven females, periods of acyclicity were more common in females that had not bred for at least seven years. Furthermore, non-proven females also scored higher body condition scores than proven females, and were less likely to exhibit regular oestrous behaviours. In males, faecal testosterone concentration was positively correlated with age, but controlling for this, proven breeders had significantly higher testosterone concentration than non-proven males. This chapter indicates that there may be intrinsic differences in reproductive hormones between breeding and non-breeding individuals, with irregular cyclicity prevalent among females, and reduced testosterone concentration in non-breeding males. Furthermore, a failure to exhibit oestrous behaviours may further limit opportunities for breeding. However, the reasons for these differences require further investigation.

- *The next aim of this thesis was to perform an exploratory analysis to investigate whether extrinsic factors relating to the captive environment were related to the observed differences in reproductive success.*

In Chapter 6, social and environmental factors, and how individuals may differ in their behavioural response to their surroundings, were compared between breeding and non-breeding individuals. Furthermore, these extrinsic factors were investigated with respect to adrenal activity, to determine whether challenges in the captive environment, or differences in how individuals respond to such challenges, may be related to disruption of reproductive function. Although there were certain elements of the social and physical environment that were correlated with adrenal activity in both male and female black rhinos, these were not related to differences in reproductive

success. Instead certain aspects of the captive environment may have led to increased adrenal activity perhaps as a result of increased activity, as opposed to chronic stressors associated with the captive environment.

Differences in how individuals respond to certain aspects of their environment, specifically towards humans and towards their physical environment, were not consistent with differences in reproductive success. However, in both males and females, individuals that were scored as being more unpredictable had higher faecal glucocorticoid concentration than those that were scored as almost always behaving the same. This suggests that there may be differences in how individuals respond to challenges in their environment, and this may be related to adrenal activity. Furthermore, non-proven females tended to be scored as more unpredictable than proven females, indicating a potential avenue for further investigation.

Differences in faecal testosterone concentration were also correlated with certain aspects of both enclosure design and the social environment, but again, these were not consistent with differences in breeding status. This suggests that the social and physical environment may not fully explain the differences observed in reproductive success between males in this population, and other factors that could underlie both testosterone concentration and differential reproductive success could be important in understanding reproductive skew amongst males.

- *As irregular cyclicity was observed to be quite prevalent in this population, and may be an important factor in understanding differential reproductive success, the final aim of this study was to investigate reproductive cyclicity within females, to investigate whether any differences in progesterone, corticosterone or oestradiol metabolite concentration were apparent between different cycle types.*

The occurrence of irregular cyclicity was investigated within a sub-set of the study females where samples were collected continuously every other day for a period of 9-15 months. In all of these females, whether proven (N=6) or non-proven breeders (N=12), a combination of regular and irregular cycles were observed during the study period. The key difference between cycle types was an increase in fGCM during irregular cycles (long, short and acyclic combined) compared to normal periods of cyclicity, and specifically increased fGCM during cycles more than 40 days in length, indicating potential disruption to normal physiology. However, what we cannot yet

determine is what may be the cause of this increase, or in fact whether the increase in fGCM is the cause, or result of this extended cycle type. Although it is feasible that adrenal activity could lead to the disruption of normal HPG activity, and could potentially result in the persistence of anovulatory follicles, this has not yet been confirmed as the cause of extended cycles in this species. However, what is apparent is that irregular cyclicity is perhaps more common than previously thought in the black rhinoceros, and could have implications for reproductive success.

However, although oestrogens were lower during periods of acyclicity and during short cycle types, indicating that this could be a valid route to investigate differences in cyclicity between females, overall the results of this aspect of the study were not conclusive. Perhaps an alternative method of measuring oestrogen concentration may be required to investigate the observed differences in expression of oestrus, as faecal measures were highly variable both within and between females, but consistent patterns could not be identified. Furthermore, the concentration of both progesterone and oestradiol metabolites during the preceding luteal phase and current follicular phase of the oestrous cycle did not appear to vary between cycle types, or between proven and non-proven females. However, the duration of the preceding luteal phase was significantly higher prior to long cycles, and significantly lower prior to both short cycles and acyclic periods, when compared to cycles of 20-40 days in length. This suggests that there may some alteration to normal physiology prior to these irregular periods of cyclicity, but the approach used here, using the average hormone concentration during the follicular or luteal phases, could not elucidate what differences may occur. Instead, it may be necessary to look at the changes in hormone concentrations that occur at critical times during the preceding cycle, to determine if differences in hormone secretion may exist either between different cycle types, or between breeding and non-breeding females.

CONCLUSIONS

The European captive population of eastern black rhinoceros, although currently self-sustaining, is performing sub-optimally both with respect to their *in situ* counterparts, and to the proposed target of 5% growth per annum. Population performance is primarily limited by sub-optimal reproduction, both in terms of individuals producing fewer calves per annum, but also due to the high degree of reproductive skew across

the population, leading to a large proportion of individuals not producing offspring and contributing genetics to future generations.

There appear to be several issues limiting reproduction in this population. Firstly, a number of different oestrous cycle types have been observed, including periods of acyclicity and oestrous cycles ranging from less than 20 days in length, to more than 40 days in length. However, similar to other studies where erratic cyclicity was observed, we have considered only those cycles between 20-40 days in length as normal oestrous cycles typical of this species (Brown et al. 2001; Garnier et al. 2002). We propose that periods of acyclicity and both the longer and shorter cycle types may be classed as irregular cyclicity, and their occurrence needs to be explained. At the very least, both acyclic periods lasting between 12-127 days, and long cycles lasting between 42-171 days as observed during this study will reduce the number of times a female is in oestrus over a given time-frame when compared to an average cycle of 27.1 ± 5 days, and therefore provide reduced opportunities for mating.

Furthermore, the results of this study also suggest that long cycles appear to be associated with increased adrenal activity, whereas periods of acyclicity are associated with reduced oestradiol concentration, when compared to the 20-40 day cycles. Although periods of irregular cyclicity have been observed in both breeding and non-breeding females, long cycles were more apparent in non-proven than proven females, and periods of acyclicity more often seen in females that have not bred for at least seven years, indicating that irregular cyclicity could therefore be related to reproductive success.

Non-proven females were also scored with higher body condition scores than proven females. Although we could not directly relate body condition to irregular cyclicity, obesity has been demonstrated by a number of other studies to be associated with reproductive failure, including extended oestrus cycle length in mares (Vick et al. 2006). In order to understand the potential causes and consequences of these different cycle types, further investigation is required. For example, a combination of longitudinal endocrine monitoring and ultrasound investigation may be beneficial to understand whether long and short oestrous cycles, and acyclic periods are indeed abnormal and potentially pathological, or whether they merely represent one of a set of normal physiological occurrences to external stimuli.

A further issue indicated by the results of this study is the inconsistent expression of overt behavioural signs of oestrus. In particular, non-proven females were less likely than proven females to exhibit regular signs of oestrus as identified by their keepers. Although this could be related to the increased incidence of long cycles, there were also some cases reported during this study period, where faecal progesterone metabolite concentration indicated that the female should have been in oestrus, but behavioural signs were not observed. This problem has important management consequences, as in this species, breeding pairs are often kept separate outside of oestrus, so a lack of overt signs may result in rhinos not being introduced at an opportune time for mating. However, although variation in faecal oestradiol concentration was observed both within and between females, no relationships could be determined either with periods of regular and irregular cyclicity, or between proven and non-proven females. An alternative technique for measuring oestrogens may prove useful in understanding why some females fail to express overt signs of behavioural oestrus, and perhaps urine measures need to be explored as a non-invasive alternative to faeces. Furthermore, a more detailed investigation within individual females that are known to show poor behavioural signs of oestrus would be beneficial, incorporating more detailed behavioural observations alongside longitudinal hormone analyses.

The intrinsic differences in reproductive hormones between breeding and non-breeding individuals were not limited to females. Breeding males in this population also exhibited higher faecal testosterone metabolite concentration than non-breeding males. However, during this initial exploratory investigation, no clear relationships could be identified with any social or environmental factors that might explain these differences. It may therefore be beneficial to investigate whether other intrinsic factors relating to quality or dominance might underlie these differences. Furthermore, we cannot yet determine the causal relationship as to whether proven males are better able to breed because their testosterone concentration is higher, or whether testosterone is increased due to their prior breeding status.

With around 40% of both males and females in the current population as yet failing to reproduce, it is sometimes unclear whether both individuals within a non-breeding pair are contributing to the failure to reproduce, or whether one individual may be reducing the reproductive potential of the other. During the course of this study, it has become apparent that some females are mated, but either fail to conceive or early pregnancy loss goes undetected (Berkeley et al. 1997; Roth 2006), whereas some

females fail to exhibit oestrus and so are not successfully introduced to a male. This latter scenario may be due to keepers not observing oestrus and so not being able to introduce individuals at the right time, or may be that when they do introduce a male and female, aggression is high and no mating occurs. There is still more we need to understand about reproduction in the black rhinoceros, not least the potential causes and long-term consequences of irregular oestrous cycles. However, hormone analysis has proven to be a very useful tool, providing an extra insight into reproductive physiology to help guide breeding management, especially useful when behavioural signs of oestrus are poor.

Although this study has not been able to fully elucidate differences in reproductive success, it has potentially ruled out certain issues and highlighted areas for further research. Extrinsic factors do not appear to explain the differences between proven and non-proven males or females, and furthermore, do not appear to be related to chronic adrenal activity in this population. However one area that we have not been able to address is whether social factors may be important to facilitate reproductive function. Indeed, little work has yet been conducted on this subject in the black rhinoceros, but it may be an interesting area for further study. Black rhinos have been described as both polygynous and polyandrous (Hutchins and Kreger 2006), with males mating with multiple females and vice versa. However, under natural conditions, black rhinos tend to be relatively solitary outside of oestrus (Goddard 1967), and with relatively poor eyesight, much of their communication is thought to be olfactory (Estes 1991), with urine spraying, dung scraping and flehmen all common behaviours (Berkeley et al. 1997; Garnier et al. 2002). It may therefore be interesting to determine whether olfactory communication could play a potential role in differential reproductive success. For instance, if a female is not cycling regularly, perhaps she may not be conveying the correct signals to the male, and this may provide one explanation for why successful introductions do not occur. Similarly, there may be signals of quality or compatibility that we do not yet understand that could help to explain why certain pairs of individuals do not breed successfully.

In summary, this study has indicated that although this population of eastern black rhinoceros is demographically self-sustaining, sub-optimal reproduction is limiting both the genetic diversity and the future potential for growth. Nearly 40% of the current population have yet to produce offspring, and intrinsic differences in reproductive hormones in both males and females may be related to differential

reproductive success. Although we have not yet been able to identify the potential cause of these differences, it is clear that irregular cyclicity and reduced testosterone concentration may be limiting reproductive success, and further investigation is required into the potential causes of these differences in reproductive hormone profiles.

This research also has implications for the population management of other taxa, as the methodology used here could be applied to other managed populations, both *in situ* and *ex situ*. Population viability analysis can be a useful tool to estimate the likely future status of a population, and can provide an indication of particular aspects of a species' biology, such as mortality or fecundity that have the most influence on the performance of a particular population (Dunham et al. 2008; Faust et al. 2006; Fernandez-Olalla et al. 2012). This allows targeted management to focus resources on those particular parameters, or sub-sets of individuals that could most benefit overall population performance. The comparison of different programs to perform these analyses has also provided a useful insight into how robust these different programs can be, as long as the data that is used to construct them is suitable. However, a thorough understanding of the data in question is essential to produce accurate predictions, rather than assuming a particular program will suit the data available. Nonetheless, when used correctly, PVA can be a very useful tool for *in situ* and *ex situ* conservation.

To provide the optimal conditions for *ex situ* or *in situ* breeding programmes, it is important to understand the physiology of a particular species, and understand what intrinsic and extrinsic factors can influence both individual and population performance. This may include ways to improve reproduction, reduce mortality, reduce behavioural abnormalities, or understand social influences such as mate choice or social suppression. All of these questions could potentially be addressed using hormone analysis (Brown 2006; Clark et al. 2011; Husak and Moore 2008; Mullner et al. 2004; Young et al. 2008), and the development of non-invasive techniques for monitoring both reproductive and adrenal hormones mean that these tools can be used on a wide variety of different taxa (Graham et al. 2001; Watson et al. 2013). Furthermore, the development of field techniques (Beehner and Whitten 2004; Freeman et al. 2010; MacDonald et al. 2008; Pappano et al. 2010; Santymire and Armstrong 2010), have made these techniques more easily applicable to free-ranging populations. The approach used here could therefore be beneficial to other species,

particularly where differences in reproductive success are observed, or where hormone analysis could prove beneficial in gaining a better understanding of species biology.

Although this study has focused on the black rhino, this approach also forms a basis for other multi-institutional studies, illustrating that longitudinal hormone analyses can be conducted successfully within coordinated captive breeding programmes, and as such can provide useful data to guide population management. Captive breeding populations are important to reinforce *in situ* conservation, and when managed cooperatively valuable information can be obtained to help increase our knowledge of species biology, which can be beneficial for both *in situ* and *ex situ* conservation of endangered species. To ensure the persistence of endangered species in the future, evidence-based population management is essential to maximise the work of global conservation efforts.

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APPENDIX 1

A.1 Laboratory Protocols

1. Wet-weight shaking extraction
2. Progesterone CL425 EIA
3. Plate map for CL425
4. Testosterone R156/7 EIA
5. Oestradiol R4972 EIA
6. Corticosterone CJM006 EIA

Wet Weight Shaking Extraction

Day 1

- Defrost faecal samples – aim for approximately 70-100 samples per day
- Create an extraction processing sheet with individual, unique sample number, date of sample and space for additional notes.
- Assign each sample an extraction vial number and label a set of extraction vials with number on both sides.
- Print labels and label two sets of plastic tubes with individual, sample number, date, institution and either faecal sample or faecal extract.
- Once samples have defrosted, break up and mix each sample by crushing bag between fingers
- When ready to extract each sample, open storage bag up fully and mix sample again with tweezers
- Weigh 0.5 g of faecal sample into small weigh boats – take a minimum of three subsections from different areas of the bag to make up the 0.5g
- Mark any unusual consistency, debris, or unusual smell (e.g. urine) on extraction sheet
- Transfer faecal material into numbered extraction vials - try to avoid leaving faecal material around the rim of the vial
- Fill a small plastic sample storage vial labelled with 'faecal sample' with remainder of mixed sample. Cap the vials and freeze
- Clean tweezers in between each sample with 30% methanol to avoid transfer of hormones between samples
- Once all samples have been weighed, use repeater to add 0.5 ml Milli-Q water and then 4.5 ml 100% methanol to every extraction vial
- Cap the vials. Vortex each tube until sample is well mixed (until all faecal material is freely mixing in the solution) ~10 seconds
- Place extraction vials in order in boxes and place on rotator to shake overnight
- Clean weigh balance and weighing area with methanol

Day 2

- In the morning remove extraction vials from rotator
- Vortex each sample
- Remove buckets from the centrifuge place extraction vials into buckets in order. Make sure centrifuge is balanced
- Centrifuge extraction vials for 20 minutes at 1800rpm (598g)
- While tubes are spinning label a set of glass tubes (16mm x125mm) with sample numbers on both sides according to the extraction sheet
- Pour off supernatant into corresponding # glass extraction tubes (16mm x125mm) and dry down supernatant in warm water bath (56°C) under air in fume cupboard
- Reconstitute in 1 ml methanol – add 1ml 100% methanol using repeater, cover each tube individually with parafilm then vortex and sonicate for 15 minutes
- Vortex then pour off each extract into plastic sample storage vials labelled 'faecal extracts'. Cap the vials and store in freezer until ready to use

Protocol for Progesterone EIA (CL425) – per plate

DAY 1:
Plate coating

- use NUNC Maxisorb plates
- antibody working dilution is 1:10,000
  - add 25µL working antibody stock (1:50, -20°C) to 5mL coating buffer
  - add 50µL per well antibody solution
  - do not coat column 1 - start at A2 and go down each column
  - tap plates gently to ensure that coating solution covers well bottom
  - label, cover with acetate plate sealer and leave overnight at 4°C
- plates can be prepared and used for up to one week

DAY 2:
Preparing reagents

- Standards
  - standard values are 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78pg/well
  - serially dilute working top standard (200pg/well, -20°C) 2-fold with 200µL EIA buffer
  - label one vial '0' and add 400µl of EIA buffer only
- Samples/controls
  - dilute faecal extracts in EIA buffer to the appropriate dilution
  - use pre-prepared synthetic C1 and C2 neat in assay
  - dilute C3 female black rhino faecal pool 1:70 in EIA buffer
- HRP
  - Pg-HRP working dilution is 1:35,000
  - check working stock for contamination before use, and remake if necessary
  - add 28.6µL working stock (1:200, 4°C) to 5mL EIA buffer in a glass beaker

Running the plate

- Plate washing
  - purge the plate washer
  - wash the plates five times with wash solution
  - blot the plates on paper towel to remove excess wash solution
  - run plate immediately
- Plate loading
  - add 50µL standard, sample, or control per well in duplicate as quickly and accurately as possible
  - immediately add 50µL per well of diluted Pg-HRP
  - cover the plates, label with the time and incubate at RT in light for 2 hours
- Plate washing
  - wash the plates 5 times with wash solution and blot dry
  - plates are fairly stable at this point and can be left until all plates are washed
- Substrate
  - prepare substrate immediately before use
  - combine 40µL H_2O_2, 125µL ABTS and 12.5mL substrate buffer **= substrate**
  - add 100µL substrate to all wells
  - cover and incubate at RT in light
- Plate reading
  - read at 405nm
  - ready when 0 wells reach between 0.8 and 1 .0 optical density

ASSAY Progesterone CL425 **SPECIES** _____

DATE COATED _____ **INSTITUTION** _____

DATE RUN _____ **ANIMAL ID** _____

DILUTION _____

	1	2	3	4	5	6	7	8	9	10	11	12
A	**NSB**	**0**	**6.25**	**100**	T1	T5	T9	T13	T17	T21	**C3***	**6.25**
B	**NSB**	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	**12.5**
C		**0.78**	**12.5**	**200**	T2	T6	T10	T14	T18	T22	T25	**25**
D		↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	**50**
E		**1.56**	**25**	**C1**	T3	T7	T11	T15	T19	T23	**0**	**100**
F		↓	↓	↓	↓	↓	↓	↓	↓	↓	**0.78**	**200**
G		**3.12**	**50**	**C2**	T4	T8	T12	T16	T20	T24	**1.56**	**0**
H		↓	↓	↓	↓	↓	↓	↓	↓	↓	**3.12**	**0**

COMMENTS _____

Stock Preparation for Progesterone EIA

Antibody
- antibody top stock (monoclonal CL425, C.J. Munro, UC Davis) is stored in the freezer
- when required, dilute CL425 at a dilution of 1:50 by adding 100µL of stock to 4.9mL coating buffer
- aliquot 100µL into 1ml eppendorf and freeze at -20°C **= working antibody stock**
- refreeze remaining top stock

HRP Conjugate
- Pg-HRP top stock (progesterone-3CMO-horseradish peroxidase; C.J. Munro, UC Davis) is stored in the freezer
- when required, dilute Pg-HRP 1:200 by adding 6.25µL stock to 1.244mL EIA assay buffer and store at 4°C **= working HRP stock**
- working HRP stock is stored in the fridge, and must be remade once sediment/contamination appears
- refreeze remaining top stock

Standards
- weigh 1mg progesterone (P0130, Sigma Aldrich) and add to 1mL EtOH for a 1mg/mL primary stock
- take 1ml primary stock (1mg/mL) and add to 99mL EtOH assay buffer for a 1mg/100mL (10,000ng/ml) secondary stock in alcohol
- standard secondary stock is stored in the fridge
- when required, dilute further (1:2500) by adding 20µL secondary stock to 49.9mL EIA buffer to prepare 200pg/well or 4pg/µl standard stock
- aliquot 4ml into 12x75 polypropylene tubes and freeze at -20°C **= working top standard**

Controls
- make C1 to bind at ~30% and C2 at ~70 % using secondary standard stock
- aliquot 3ml into 12x75 polypropylene tubes, label and freeze at -20°C

Protocol for Testosterone EIA (R156/7) – per plate

DAY 1:

Plate coating with non-specific anti-rabbit IgG

- use NUNC Maxisorb plates
- coat plate with anti-rabbit IgG (1.0µg/well), including blank/NSBs
  - add 500µL working IgG (1mg/5ml, -20°C) to 25mL coating buffer
  - add 250µL IgG solution per well
  - coat NSBs (A1/B1), do not coat remainder of column 1 – re-start at A2 and go down each column
  - tap plates gently to ensure that coating solution covers well bottom
  - label, cover with acetate plate sealer and leave overnight at room temperature (RT)

DAY 2:

Plate blocking

- empty excess IgG solution into sink with good shake and bang on paper towel, do not wash
- add 300ul blocking buffer per well (including NSBs)
- incubate at RT for a minimum of 2 hours (or can be stored like this for maximum 1 week)

Preparing reagents

- Standards
  - standard values are 600, 300, 150, 75, 37.5, 18.8, 9.4, 4.7, and 2.3 pg/well
  - serially dilute working top standard (600pg/well, -20°C) 2-fold with 200µL EIA buffer
  - label one vial '0' and add 400µl of EIA buffer only
- Samples/controls
  - dilute faecal extracts in EIA buffer to the appropriate dilution
  - use pre-prepared synthetic C1 and C2 neat in assay
  - dilute C4 male black rhino faecal pool 1:20 in EIA buffer
- HRP
  - Tt-HRP working dilution is 1:45,000
  - check working stock for contamination before use, and remake if necessary
  - add 11.1µL working stock (1:100, 4°C) to 5mL EIA buffer in a glass beaker
- Antibody (AB)
  - Tt-AB working dilution is 1:25,000
  - add 20µL working stock (1:100, -20°C) to 5mL EIA buffer in a glass beaker

Running the plate

- Plate washing
  - purge the plate washer
  - wash the plates five times with wash solution
  - blot the plates on paper towel to remove excess wash solution
  - run plate immediately
- Plate loading – follow plate map
  - add 50µL EIA buffer per well
  - add 50µL standard, sample, or control per well in duplicate as quickly and accurately as possible
  - add 50µL per well of diluted Tt-HRP
  - add 50µL per well of diluted Tt-AB (Do not add to NSBs)
  - cover the plates, label with the time and incubate at RT in dark for 2 hours
- Plate washing
  - wash the plates 5 times with wash solution and blot dry
  - plates are fairly stable at this point and can be left until all plates are washed

Substrate

- prepare substrate immediately before use
- combine 40µL H_2O_2, 125µL ABTS and 12.5mL substrate buffer **= substrate**
- add 100µL substrate to all wells
- cover and incubate at RT in dark

Plate reading

- read at 405nm
- ready when 0 wells reach between 0.8 and 1 .0 optical density

Stock Preparation for Testosterone EIA

Non-specific goat anti-rabbit IgG

- dissolve 1mg anti-rabbit IgG (R2004, Sigma Aldrich) in 5ml coating buffer
- use a 5ml syringe and needle to piercing rubber stopper to avoid loss of powder
- gently shake to dissolve powder until solution becomes hazy, not stringy
- solution will appear clear at first, becoming stringy then hazy
- can take several hours so place on orbital shaker on low speed
- freeze at -20°C = **working IgG**

Antibody

- antibody top stock (polyclonal R156/7, C.J. Munro, UC Davis) is stored in the freezer
- when required, dilute R156/7 at a dilution of 1:100 by adding 20µL of stock to 2.0mL coating buffer
- aliquot 100µL into 1ml eppendorf and freeze at -20°C **= working antibody stock**
- refreeze remaining top stock

HRP Conjugate

- Tt-HRP top stock (testosterone-horseradish peroxidase; C.J. Munro, UC Davis) is stored in the freezer
- when required, dilute Tt-HRP 1:100 by adding 12.5µL stock to 1.238mL EIA assay buffer and store at 4°C **= working HRP stock**
- working HRP stock is stored in the fridge, and must be remade once sediment/contamination appears
- refreeze remaining top stock

Standards

- weigh 1mg testosterone (T1500, Sigma Aldrich) and add to 1mL EtOH for a 1mg/mL primary stock
- take 1ml primary stock (1mg/mL) and add to 99mL EtOH assay buffer for a 1mg/100mL (10,000ng/ml) secondary stock in alcohol
- standard secondary stock is stored in the fridge
- when required, dilute further (1:833) by adding 60µL secondary stock to 50mL EIA buffer to prepare 600pg/well or 12pg/µl standard stock
- aliquot 4ml into 12x75 polypropylene tubes and freeze at -20°C **= working top standard**

Controls

- make C1 to bind at ~30% and C2 at ~70 % using secondary standard stock
- aliquot 3ml into 12x75 polypropylene tubes, label and freeze at -20°C

Protocol for Oestradiol EIA (R4972) – per plate

DAY 1:

Plate coating

- use NUNC Maxisorb plates
- antibody working dilution is 1:20,000
  - add 25µL working antibody stock (1:100, -20°C) to 5mL coating buffer
  - add 50µL per well antibody solution
  - do not coat column 1 - start at A2 and go down each column
  - tap plates gently to ensure that coating solution covers well bottom
  - label, cover with acetate plate sealer and leave overnight at 4°C
- plates can be prepared and used for up to one week

DAY 2:

Plate washing

- purge the plate washer
- wash the plates five times with wash solution
- blot the plates on paper towel to remove excess wash solution
- run plate immediately

Buffer plates

- add 50µl of EIA assay buffer to each well (including blanks) – incubate at RT 1-5 hours

Preparing reagents

- Standards
  - standard values are 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 1.95 pg/well
  - serially dilute working top standard (500pg/well, -20°C) 2-fold with 100µL EIA buffer
  - label one vial '0' and add 200µl of EIA buffer only
- Samples/controls
  - dilute faecal extracts in EIA buffer to the appropriate dilution
  - use pre-prepared synthetic C1 and C2 neat in assay
  - dilute C3 female black rhino faecal pool 1:50 in EIA buffer
- HRP
  - E2-HRP working dilution is 1:65,000
  - check working stock for contamination before use, and remake if necessary
  - add 38.5µL working stock (1:500, 4°C) to 5mL EIA buffer in a glass beaker

Running the plate

- Plate loading
  - add 20µL standard, sample, or control per well in duplicate as quickly and accurately as possible
  - immediately add 50µL per well of diluted E2-HRP
  - cover the plates, label with the time and incubate at RT in light for 2 hours
- Plate washing
  - wash the plates 5 times with wash solution and blot dry
  - plates are fairly stable at this point and can be left until all plates are washed

Substrate

- prepare substrate immediately before use
- combine 40µL H_2O_2, 125µL ABTS and 12.5mL substrate buffer **= substrate**
- add 100µL substrate to all wells
- cover and incubate at RT in light

Plate reading

- read at 405nm
- ready when 0 wells reach between 0.8 and 1 .0 optical density

Stock Preparation for Oestradiol EIA

Antibody

- antibody top stock (polyclonal R4972, C.J. Munro, UC Davis) is stored in the freezer
- when required, dilute R4972 at a dilution of 1:100 by adding 50µL of stock to 4.95mL coating buffer
- aliquot 200µL into 1ml eppendorf and freeze at -20°C **= working antibody stock**
- refreeze remaining top stock

HRP Conjugate

- CC-HRP top stock (E2-17beta horseradish peroxidase; C.J. Munro, UC Davis) is stored in the freezer
- when required, dilute E2-HRP 1:500 by adding 5µL stock to 2.475mL EIA assay buffer and store at 4°C **= working HRP stock**
- working HRP stock is stored in the fridge, and must be remade once sediment/contamination appears
- refreeze remaining top stock

Standards

- weigh 1mg estradiol-17β (E8875, Sigma Aldrich) and add to 1mL EtOH for a 1mg/mL primary stock
- take 1ml primary stock (1mg/mL) and add to 99mL EtOH assay buffer for a 1mg/100mL (10,000ng/ml) secondary stock in alcohol
- standard secondary stock is stored in the fridge
- when required, dilute further (1: 400) by adding 100µL secondary stock to 39.9mL EIA buffer to prepare 500pg/well or 25ng/ml standard stock
- aliquot 2ml into 12x75 polypropylene tubes and freeze at -20°C = **working top standard**

Controls

- make C1 to bind at ~30% and C2 at ~70 % using secondary standard stock
- aliquot 2ml into 12x75 polypropylene tubes, label and freeze at -20°C

Protocol for Corticosterone EIA (CJM006) – per plate

DAY 1:

Plate coating

- use NUNC Maxisorb plates
- antibody working dilution is 1:15,000
  - add 33.3µL working antibody stock (1:100, -20°C) to 5mL coating buffer
  - add 50µL per well antibody solution
  - do not coat column 1 - start at A2 and go down each column
  - tap plates gently to ensure that coating solution covers well bottom
  - label, cover with acetate plate sealer and leave overnight at 4°C
- Plates are <u>not</u> ready to use the following day (day 2), but can be used on <u>day 3, 4 or 5</u>

DAY 3:

Preparing reagents

- Standards
  - standard values are 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9 pg/well
  - serially dilute working top standard (1000pg/well, -20°C) 2-fold with 200µL EIA buffer
  - label one vial '0' and add 400µl of EIA buffer only
- Samples/controls
  - dilute faecal extracts in EIA buffer to the appropriate dilution
  - use pre-prepared synthetic C1 and C2 neat in assay
  - dilute C3 female black rhino faecal pool 1:20 in EIA buffer
- HRP
  - CC-HRP working dilution is 1:70,000
  - Check working stock for contamination before use, and remake if necessary
  - add 7.14µL working stock (1:100, 4°C) to 5mL EIA buffer in a glass beaker

Running the plate

- Plate washing
  - purge the plate washer
  - wash the plates five times with wash solution
  - blot the plates on paper towel to remove excess wash solution
  - run plate immediately
- Plate loading
  - add 50µL standard, sample, or control per well in duplicate as quickly and accurately as possible
  - immediately add 50µL per well of diluted CC-HRP
  - cover the plates, label with the time and incubate at RT in dark for 2 hours
- Plate washing
  - wash the plates 5 times with wash solution and blot dry
  - plates are fairly stable at this point and can be left until all plates are washed

Substrate

- prepare substrate immediately before use
- combine 40µL H2O2, 125µL ABTS and 12.5mL substrate buffer **= substrate**
- add 100µL substrate to all wells
- cover and incubate at RT in dark

Plate reading

- read at 405nm
- ready when 0 wells reach between 0.8 and 1 .0 optical density

Stock Preparation for Corticosterone EIA

Antibody

- antibody top stock (polyclonal CJM006, C.J. Munro, UC Davis) is stored in the freezer
- when required, dilute CJM006 at a dilution of 1:100 by adding 20µL of stock to 2000µL coating buffer
- aliquot 100µL into 1ml eppendorf and freeze at -20°C **= working antibody stock**
- refreeze remaining top stock

HRP Conjugate

- CC-HRP top stock (corticosterone-3CMO-horseradish peroxidase ; C.J. Munro, UC Davis) is stored in the freezer
- when required, dilute CC-HRP 1:100 by adding 25µL stock to 2.475mL EIA assay buffer and store at 4°C = **working HRP stock**
- working HRP stock is stored in the fridge, and must be remade once sediment/contamination appears
- refreeze remaining top stock

Standards

- weigh 1mg corticosterone (C2505, Sigma Aldrich) and add to 1mL EtOH for a 1mg/mL primary stock
- take 1ml primary stock (1mg/mL) and add to 99mL EtOH assay buffer for a 1mg/100mL (10,000ng/ml) secondary stock in alcohol
- standard secondary stock is stored in the fridge
- when required, dilute further (1: 500) by adding 100µL secondary stock to 49.9mL EIA buffer to prepare 1000pg/well or 20ng/ml standard stock
- aliquot 4ml into 12x75 polypropylene tubes and freeze at -20°C = **working top standard**

Controls

- make C1 to bind at ~30% and C2 at ~70 % using secondary standard stock
- aliquot 3ml into 12x75 polypropylene tubes, label and freeze at -20°C

APPENDIX 2

A.2 Enzyme immunoassay antibody cross-reactivities

Table A.2.1: Cross-reactivity of progesterone antibody (CL425) to various progesterone (P4) metabolites, relative to the binding of progesterone.

Progesterone metabolite	Common name	Cross-reactivity (%)
4-Pregnen-3,20-dione	Progesterone	100.0
4-Pregnen-3α-o1-20-one		188.0
4-Pregnen-3β-o1-20-one		172.0
4-Pregnen-11α-o1-3,20-dione		147.0
5α-Pregnan-3β-o1-20-one		94.0
5α-Pregnan-3α-o1-20-one		64.0
5α-Pregnan-3,20-dione		55.0
5β-Pregnan-3β-o1-20-one		12.5
5β-Pregnan-3,20-dione		8.0
4-Pregnen-11β-o1-3,20-dione		2.7
5β-Pregnan-3α-o1-20-one		2.5
5β-Pregnan-3α,20α-diol	Pregnanediol	<0.1
5α-Pregnan-3α,20β-diol		<0.1
5β-Pregnan-3,17-dione	Androstenedione	<0.1
5β-Pregnan-11β,21-diol-3,20-dione	Corticosterone	<0.1

Table A.2.2: Cross-reactivity of corticosterone antibody (CJM006) to various compounds, relative to the binding of corticosterone.

Common name	Cross-reactivity (%)
Corticosterone	100.00
Desoxycorticosterone	14.25
Progesterone	2.65
Tetrahydrocorticosterone	0.90
Testosterone	0.64
Cortisol	0.23
Prednisolone	0.07
11-Desoxycortisol	0.03
Prednisone	<0.01
Cortisone	<0.01
Estradiol-17β	<0.01

Table A.2.3: Cross-reactivity of oestradiol antibody (R4972) to various compounds, relative to the binding of oestradiol-17β.

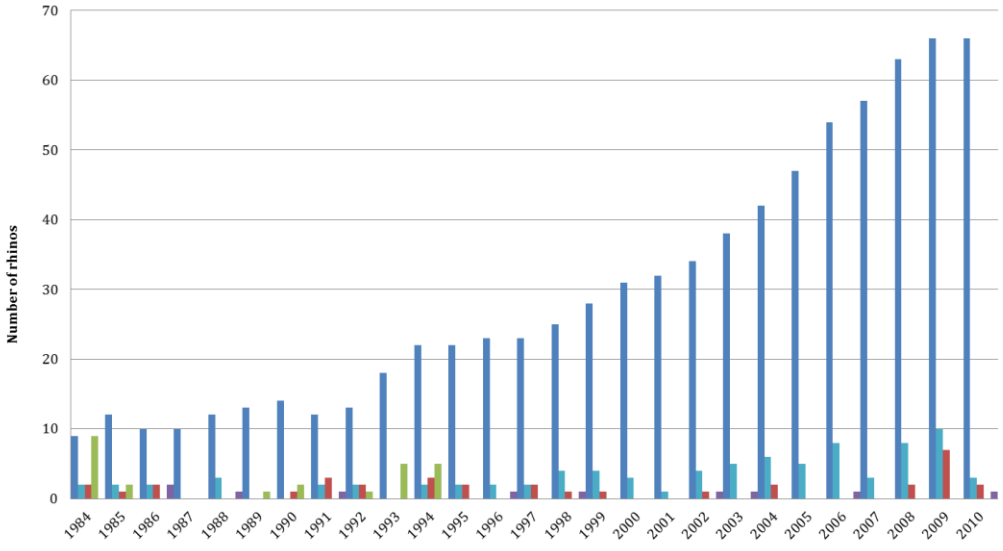
Common name	Cross-reactivity (%)
Estradiol-17β	100.0
Estrone	3.3
Testosterone	1.0
Progesterone	0.8
Estrone sulfate	<0.1
Cortisol	<0.1
Corticosterone	<0.1
Androstenedione	<0.1

Table A.2.4: Cross-reactivity of testosterone antibody (R156/7) to various compounds, relative to the binding of testosterone.

Common name	Cross-reactivity (%)
Testosterone	100.00
5α-Dihydrotestosterone	57.37
Androstenedione	0.27
Androsterone	0.04
DHEA	0.04
Cholesterol	0.03
β-Oestradiol	0.02
Progesterone	<0.02
Pregnenolone	<0.02
Hydrocortisone	<0.02
Cholic acid	<0.02
Chenodeoxycholic acid	<0.02
Cholic acid methyl ester	<0.02
Dehydrocholic acid	<0.02
Deoxycholic acid	<0.02
Lithocholic acid	<0.02
Glycholic acid	<0.02
Taurodeoxycholic acid	<0.02
Taurocholic acid	<0.02
Taurochenodeoxycholic acid	<0.02
Glycochenodeoxycholic acid	<0.02

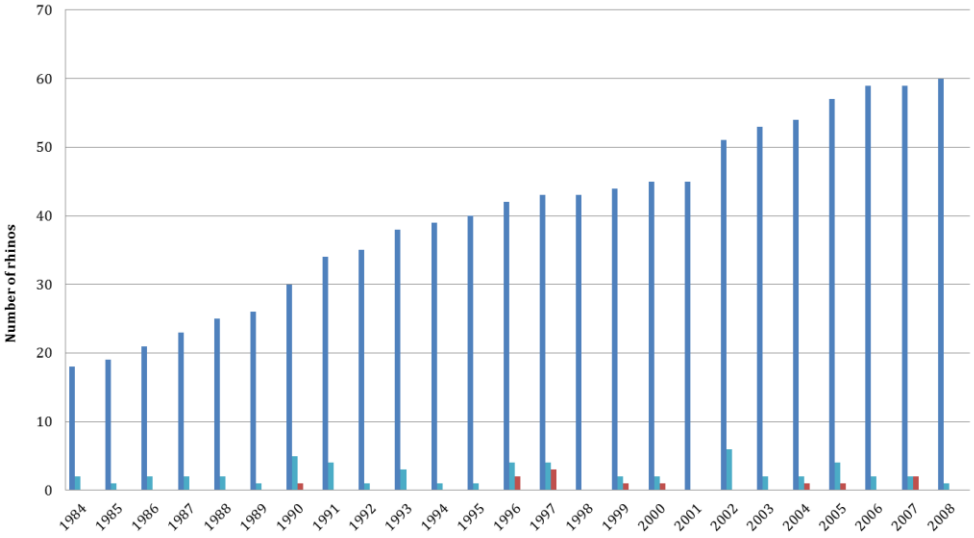
APPENDIX 3

A.3 Population trends of eastern black rhino at eight Kenyan reserves during the recording period



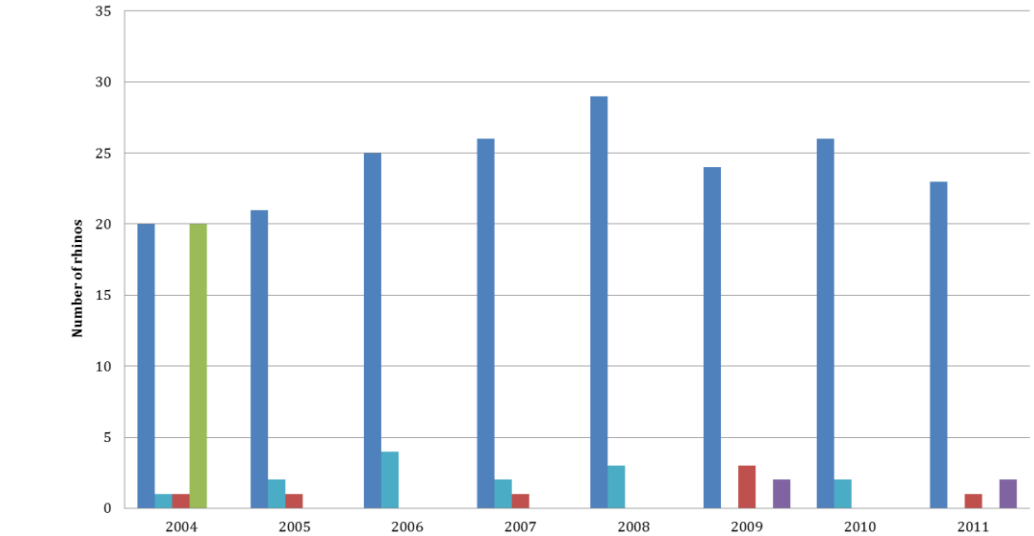
	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Total population	9	12	10	10	12	13	14	12	13	18	22	22	23	23	25	28	31	32	34	38	42	47	54	57	63	66	66
Births	2	2	2	0	3	0	0	2	2	0	2	2	2	2	4	4	3	1	4	5	6	5	8	3	8	10	3
Deaths	2	1	2	0	0	0	1	3	2	0	3	2	0	2	1	1	0	0	1	0	2	0	0	0	2	7	2
Imports	9	2	0	0	0	1	2	0	1	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exports	0	0	2	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0	0	1

Figure A.3.1: Population trend of black rhinoceros in Lewa Downs Conservancy between establishment in 1984 and 2010 representing total population size, births, deaths, imports and exports.



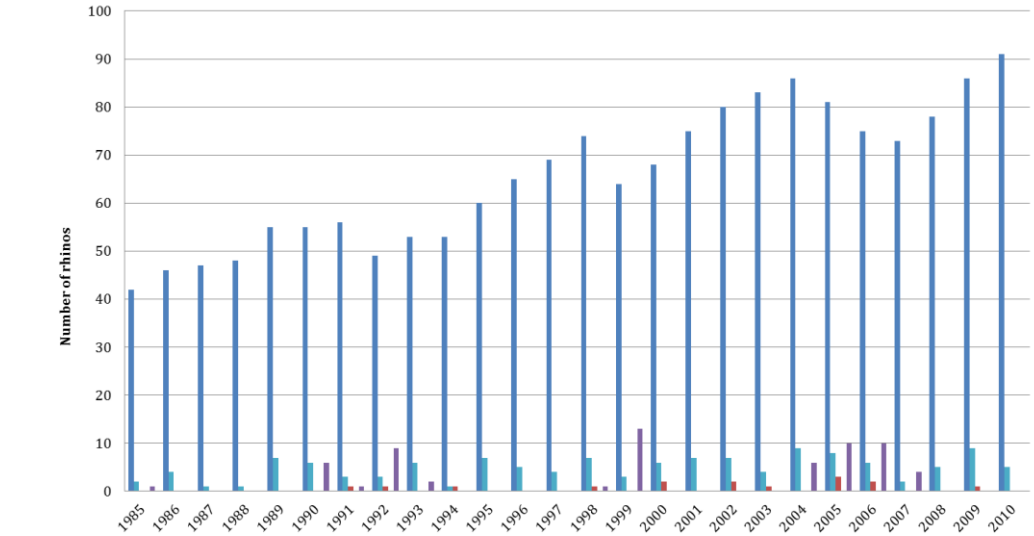
	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
Total population	18	19	21	23	25	26	30	34	35	38	39	40	42	43	43	44	45	45	51	53	54	57	59	59	60
Births	2	1	2	2	2	1	5	4	1	3	1	1	4	4	0	2	2	0	6	2	2	4	2	2	1
Deaths	0	0	0	0	0	0	1	0	0	0	0	0	2	3	0	1	1	0	0	0	1	1	0	2	0
Imports	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exports	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure A.3.2: Population trend of black rhinoceros in Masai Mara Nature Reserve between 1984 and 2008 representing total population size, births, deaths, imports and exports.



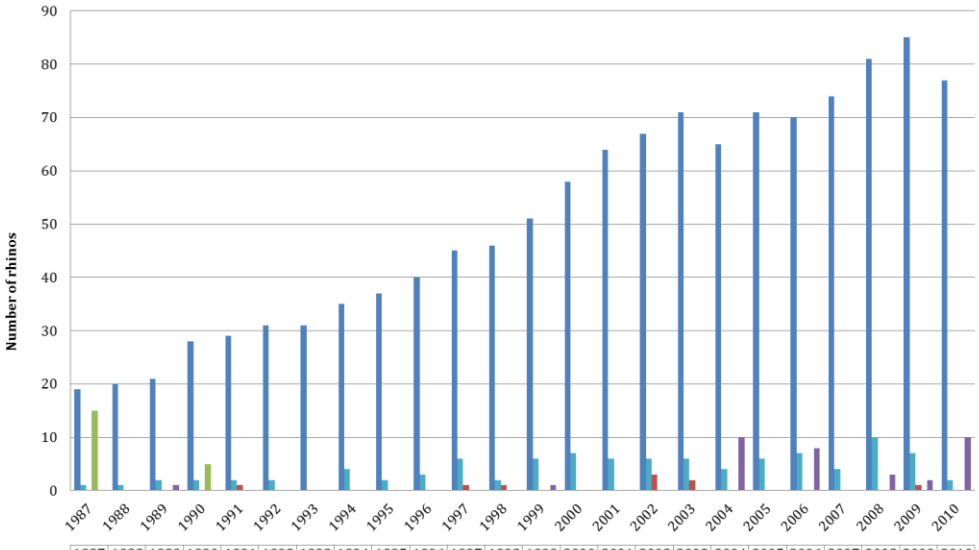
	2004	2005	2006	2007	2008	2009	2010	2011
Total population	20	21	25	26	29	24	26	23
Births	1	2	4	2	3	0	2	0
Deaths	1	1	0	1	0	3	0	1
Imports	20	0	0	0	0	0	0	0
Exports	0	0	0	0	0	2	0	2

Figure A.3.3: Population trend of black rhinoceros in Mugie Rhino Sanctuary between establishment in 2004 and 2010 representing total population size, births, deaths, imports and exports.



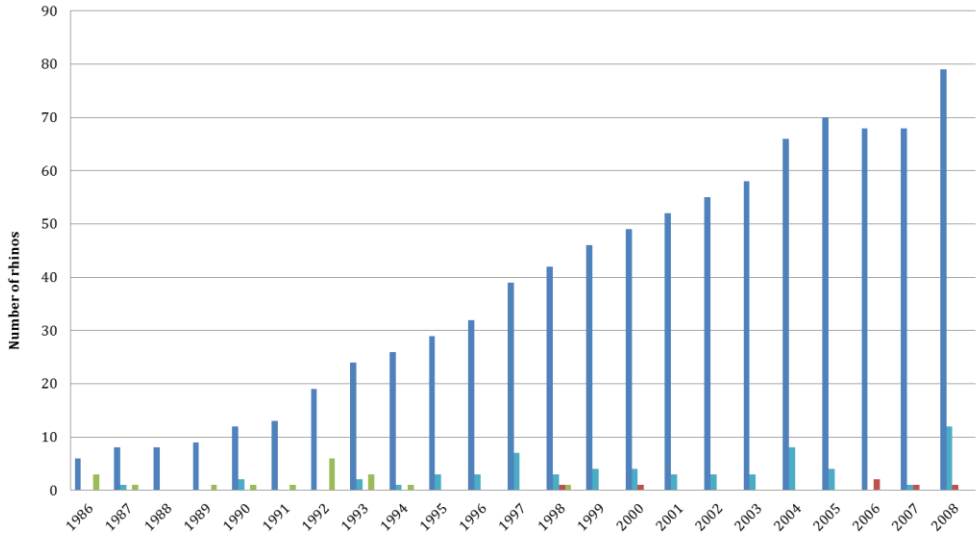
	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Total population	42	46	47	48	55	55	56	49	53	53	60	65	69	74	64	68	75	80	83	86	81	75	73	78	86	91
Births	2	4	1	1	7	6	3	3	6	1	7	5	4	7	3	6	7	7	4	9	8	6	2	5	9	5
Deaths	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	2	0	2	1	0	3	2	0	0	1	0
Imports	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exports	1	0	0	0	0	6	1	9	2	0	0	0	0	1	13	0	0	0	0	6	10	10	4	0	0	0

Figure A.3.4: Population trend of black rhinoceros in Nairobi National Park between 1985 and 2010 representing total population size, births, deaths, imports and exports.



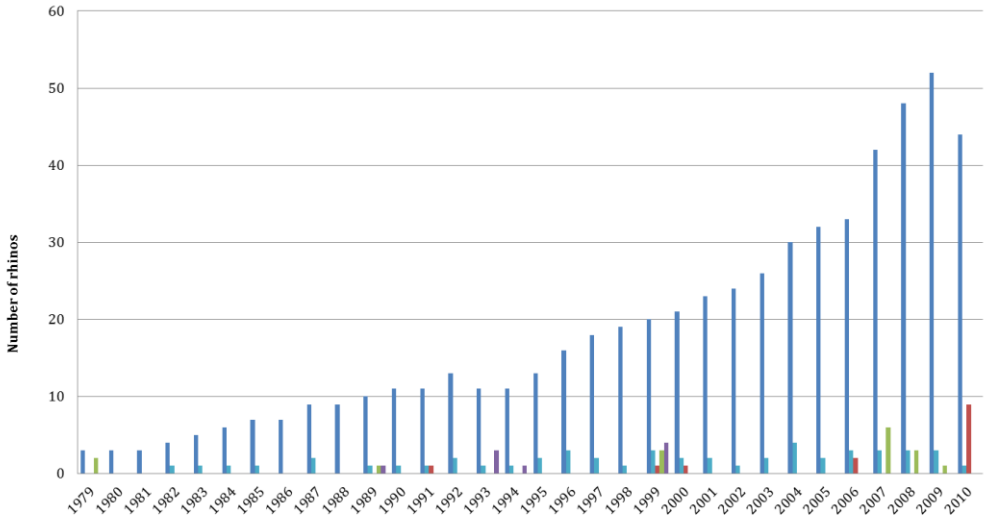
	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Total population	19	20	21	28	29	31	31	35	37	40	45	46	51	58	64	67	71	65	71	70	74	81	85	77
Births	1	1	2	2	2	2	0	4	2	3	6	2	6	7	6	6	6	4	6	7	4	10	7	2
Deaths	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	3	2	0	0	0	0	0	1	0
Imports	15	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exports	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	10	0	8	0	3	2	10

Figure A.3.5: Population trend of black rhinoceros in Lake Nakuru National Park between establishment in 1987 and 2010; representing total population size, births, deaths, imports and exports.



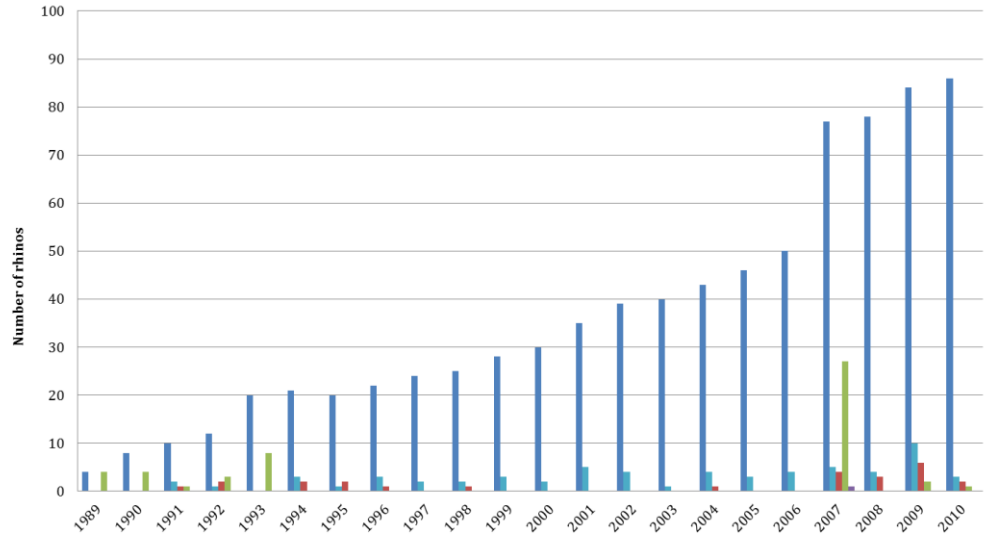
	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
Total population	6	8	8	9	12	13	19	24	26	29	32	39	42	46	49	52	55	58	66	70	68	68	79
Births	0	1	0	0	2	0	0	2	1	3	3	7	3	4	4	3	3	3	8	4	0	1	12
Deaths	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	2	1	1
Imports	3	1	0	1	1	1	6	3	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Exports	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure A.3.6: Population trend of black rhinoceros in Ngulia Rhino Sanctuary between establishment in 1986 and 2008 representing total population size, births, deaths, imports and exports.



	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Total population	3	3	3	4	5	6	7	7	9	9	10	11	11	13	11	11	13	16	18	19	20	21	23	24	26	30	32	33	42	48	52	44
Births	0	0	0	1	1	1	1	0	2	0	1	1	1	2	1	1	2	3	2	1	3	2	2	1	2	4	2	3	3	3	3	1
Deaths	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2	0	0	0	9
Imports	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	6	3	1	0
Exports	0	0	0	0	0	0	0	0	0	0	1	0	0	0	3	1	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0

Figure A.3.7: Population trend of black rhinoceros in Ol Jogi Conservancy between establishment in 1979 and 2010 representing total population size, births, deaths, imports and exports.



	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Total population	4	8	10	12	20	21	20	22	24	25	28	30	35	39	40	43	46	50	77	78	84	86
Births	0	0	2	1	0	3	1	3	2	2	3	2	5	4	1	4	3	4	5	4	10	3
Deaths	0	0	1	2	0	2	2	1	0	1	0	0	0	0	0	1	0	0	4	3	6	2
Imports	4	4	1	3	8	0	0	0	0	0	0	0	0	0	0	0	0	0	27	0	2	1
Exports	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Figure A.3.8: Population trend of black rhinoceros in Ol Pejeta Conservancy between establishment in 1989 and 2010 representing total population size, births, deaths, imports and exports.

APPENDIX 4

A.4 MATLAB code

Stochastic simulation based on vital rates, adapted from Morris and Doak (*2002*)

```
%VitalSim:
% This is a program to do a stochastic PVA simulation

clear all;

%*****************Simulation Parameters**********************
% the data shown here are for the European captive population of
Diceros bicornis michaeli, using data from 1986-2010:
% the parameters are defined in a set of arrays
% with elements corresponding to these vital rates:
%1,   2,    3,    4,    5,    6     7     8     9     10    11    12
%vrf1 vrf2  vrf3  vrf4  vrf5  vrf6  vrs1  vrs2  vrs3  vrs4  vrs5
vrs6
%13    14    15    16    17    18
%vrg1  vrg2  vrg3  vrg4  vrg5  vrg6

% vrtypes identifies the distribution for each rate:
% 1 = beta, 2 = stretched beta, 3 = lognormal
vrtypes= [ones(1,18)];
% now means and variances of these rates or elements
vrmeans= [0.0000 0.0000 0.0664 0.1029 0.0768 0.0000 0.8718 0.9921
0.9832 0.9879 0.9748 0.9465 1.0000 0.2486 0.2337 0.1142 0.0455
0.0000];
vrvars= [0.0000 0.0000 0.0076 0.0141 0.0048 0.0000 0.0447 0.0009
0.0053 0.0007 0.0019 0.0249 0.0000 0.0197 0.0231 0.0119 0.0047
0.0000];
% minimum and maximum values for each vital rate:
% put in zeros for rates that are not stretched betas
vrmins=zeros(1,18); vrmaxs=zeros(1,18);
np = length(vrmeans); % how many parameters are there?

% you must also have a separate m-file that uses a vector of vital
% rates (vrs) of the same form as vrmeans, above, to define the
% elements of the populaton matrix (mx)
makemx='KEmxdef'; % KEmxdef.m defines the elements of mx

n0=[2; 8; 7; 16; 14; 4];% initial population vector
Nx = 20;    % quasi-extinction threshold
tmax = 10;  % number of years to simulate;
np  = 0;    % number vital rates in correlation matrix;
np2 = 18;   % number of uncorrelated rates.
dims = 6;   % dimensions of the population matrix;
runs = 1000; % how many trajectories to do
%************************************************************

randn('state',sum(100*clock)); % seeds random numbers
Nstart = sum(n0); % starting population number
vrs=vrmeans;    % set vital rates to their mean values
eval(makemx);   % use matrix definition file to make mean matrix
lam0=max(eig(mx)); %find the deterministic population growth rate

%------------------------------------------------------------
% this section makes sets of beta or str. beta values to choose
% from during the simulations; it makes 99 values for 1%
```

```
% increments of Fx for each parameter -- if you already have made
% a set of beta values for this life history, you can recall
%  these, and save the time of recalculating them.
yesno = ...
input('type 0 to calculate betas, or 1 to get a stored set');
if yesno == 1
betafile = ...
input('type filename with stored betas; put in single quotes');
load(betafile)% this line is corrected from that in Morris and Doak
else    % make a set of values for each beta or stretched beta
parabetas=zeros(99,np+np2);
for ii = 1:(np+np2)
         if vrtypes(ii) ~= 3
         for fx99 = 1:99
         if vrtypes(ii) ==1;
parabetas(fx99,ii) = ...
betaval(vrmeans(ii),sqrt(vrvars(ii)),fx99/100); end;
         if vrtypes(ii) ==2;
parabetas(fx99,ii) = ...
stretchbetaval(vrmeans(ii),sqrt(vrvars(ii)),...
vrmins(ii), vrmaxs(ii), fx99/100); end;
         end; % fx99
         end; % if vrtypes(ii)
end; % ii loop
yesno = input('type 1 to store the betas, or 0 if not');
if yesno ==1
         betafile = ...
         input('type filename to store betas, put in single
         quotes');
         save(betafile, 'parabetas'); % this line is corrected from
         that in Morris and Doak
end; %if yesno
end; %else

% finally, do sets of runs to get growth rate and extinction risk
results = []; normresults = [];
PrExt = zeros(tmax,1);    % the extinction time tracker
logLam = zeros(runs,1);   % the tracker of log-lambda values
stochLam = zeros(runs,1); % tracker of stochastic lambda values
for xx = 1:runs;
if round(xx/10) == xx/10 disp(xx); end; % displays progress
nt = n0; % start at initial population vector
extinct = 0;
for tt = 1:tmax

         yrxy=[randn(np2,1)];
         %adds in randoms for uncorrelated vital rates.
         for yy = 1:(np+np2)  % loop finds vital rate values
         if vrtypes(yy) ~= 3 % if not a lognormal rate
index = round(100*stnormfx(yrxy(yy)));
if index == 0 index = 1; end; % round at extremes
if index ==100 index = 99; end;
vrs(yy) = parabetas(index,yy); % find stored value
         % else, calculated a lognormal value:
         else vrs(yy) = lnorms(vrmeans(yy),vrvars(yy),yrxy(yy));
         end;% if vrtypes(yy) ~= 3
         end; % yy loop
         eval(makemx);   % make a matrix with the new vrs values.
         nt = mx*nt;     % multiply by the population vector

         if extinct == 0 % check for extinction
```

```
          Ntot (xx,tt)= sum(nt);
          if Ntot <= Nx
          PrExt(tt) = PrExt(tt) +1;
          extinct = 1;
          end; % if Ntot
          end; % if extinct

end % time (tt) loop
logLam(xx) = (1/tmax)*log(sum(nt)/Nstart); % calculate loglambda
stochLam(xx) = (sum(nt)/Nstart)^(1/tmax);   % and stoch. lambda
end; % runs (xx) loop

CDFExt = cumsum(PrExt./runs); % make the extinction CDF function
disp(' for the last 25 years studbook data');
disp('This is the deterministic lambda value'); disp(lam0);
disp('And this is the mean stochastic lambda');
disp(exp(mean(logLam)));
disp('Below are mean and standard deviation of log lambda');
disp(mean(logLam)); disp(std(logLam));
disp('Next is a histogram of logLams'); hist(logLam);
disp('And now, the extintion time CDF'); figure; plot(CDFExt);

MeanN=mean(Ntot);
MedianN=median(Ntot);
MeanPrExt=mean(PrExt);

plot(mean(Ntot), 'DisplayName', 'Ntot', 'YDataSource', 'Ntot');
figure(gcf)
axis ([0 tmax 0 60]);
```

Calls for 'KEmxdef.m'

```
mx=[...
          vrs(1)*vrs(7)^(1/2)*vrs(7)^(1/2)
          vrs(2)*vrs(7)^(1/2)*vrs(8)^(1/2)
          vrs(3)*vrs(7)^(1/2)*vrs(9)^(1/2)...
          vrs(4)*vrs(7)^(1/2)*vrs(10)^(1/2)
          vrs(5)*vrs(7)^(1/2)*vrs(11)^(1/2)
          vrs(6)*vrs(7)^(1/2)*vrs(12)^(1/2);
          vrs(7)*vrs(13)  vrs(8)*(1-vrs(14))   0   0   0   0;
          0   vrs(8)*vrs(14)  vrs(9)*(1-vrs(15))    0   0   0;
          0   0   vrs(9)*vrs(15)  vrs(10)*(1-vrs(16)) 0   0;
          0   0   0   vrs(10)*vrs(16) vrs(11)*(1-vrs(17)) 0;
          0    0   0   0   vrs(11)*vrs(17) vrs(12)*(1-vrs(18))];
```

Calls for "eigenall.m' from Morris and Doak (2002)

```
function [lambdas,lambda1,W,w,V,v]=eigenall(A);
%   [lambdas,lambda1,W,w,V,v]=eigenall(A)
%   takes the projection matrix A as the argument of the function
%   eigenall and returns:
%      lambdas, a vector containing the eigenvalues of A;
%      lambda1, the dominant eigenvalue of A;
%      W, a matrix with the right eigenvectors of A as its columns;
%      w, the dominant right eigenvector of A (rescaled to
proportions);
%      V, a matrix with the left eigenvectors of A as its rows; and
%      v, the dominant left eigenvector of A (rescaled as multiples
of
%                                              its first element).
%   Eigenvalues and eigenvectors are sorted from largest
%   to smallest.

[W,lambdas]=eig(A);          % W=matrix with right eigenvectors of A
                             %       as columns
V=conj(inv(W));              % V=matrix with left eigenvectors of A
                             %       as rows
lambdas=diag(lambdas);       % lambdas=vector of eigenvalues
[lambdas,I]=sort(lambdas);   % sort eigenvalues from smallest to
                             %       largest
lambdas=flipud(lambdas);     % flip lambdas so that largest value
                             %       comes first
lambda1=lambdas(1);          % lambda1=dominant eigenvalue
I=flipud(I);                 % flip the index vector I
W=W(:,I);                    % sort right eigenvectors
V=V(I,:);                    % sort left eigenvectors
w=W(:,1);                    % w=stable distribution
w=w/sum(w);                  % rescale w to represent proportions
v=real(V(1,:))';             % v=vector of reproductive values
v=v/v(1);                    % rescale v relative to class 1


disp('lambda1');
disp(lambda1)
disp('lambdas');
disp(lambdas)
disp('w');
disp(w)
disp('v');
disp(v)
```

Variance stabilised sensitivity, adapted from Morris and Doak (*2002*) *and Link and Doherty* (*2002*).

```
% Vitalsens.m  Takes vital rate means and a matrix
% definition to calculate deterministic sensitivities and
% elasticities of lambda to vital rates, using symbolic
% functions to take derivatives.
% The program calls the function eigenall.m (Box 7.1)

%IMPORTANT NOTE: you can't run this program without having
% the Symbolic toolbox for Matlab
% this program was changed on 4/33/04 to conform to newer MATLAB
conventions
clear all;
%**************** SIMULATION PARAMETERS *********************
vr = [0.0000 0.0000 0.0664 0.1029 0.0768 0.0000 0.8718 0.9921 0.9832
0.9879 0.9748 0.9465 1.0000 0.2486 0.2337 0.1142 0.0455 0.0000];  %
vital rates
vrvar = [0.0000 0.0000 0.0076 0.0141 0.0048 0.0000 0.0447 0.0009
0.0053 0.0007 0.0019 0.0249 0.0000 0.0197 0.0231 0.0119 0.0047
0.0000];
syms  vrf1 vrf2 vrf3  vrf4  vrf5  vrf6  vrs1  vrs2  vrs3  vrs4  vrs5
vrs6  vrg1  vrg2  vrg3  vrg4  vrg5  vrg6  % vital rates as symbolic
variables
Svr = [vrf1 vrf2 vrf3 vrf4 vrf5 vrf6 vrs1 vrs2 vrs3 vrs4 vrs5 vrs6
vrg1 vrg2 vrg3 vrg4 vrg5 vrg6]; % vector of symbolic vital rates

% Next, a symbolic definition of the matrix
mx =    [vrf1*vrs1^(1/2)*vrs1^(1/2)    vrf2*vrs1^(1/2)*vrs2^(1/2)
vrf3*vrs1^(1/2)*vrs3^(1/2)...
         vrf4*vrs1^(1/2)*vrs4^(1/2)   vrf5*vrs1^(1/2)*vrs5^(1/2)
         vrf6*vrs1^(1/2)*vrs6^(1/2);
         vrs1*vrg1  vrs2*(1-vrg2)   0   0   0   0;
         0   vrs2*vrg2  vrs3*(1-vrg3)    0   0   0;
         0   0   vrs3*vrg3  vrs4*(1-vrg4) 0   0;
         0   0   0   vrs4*vrg4 vrs5*(1-vrg5) 0;
         0   0   0   0   vrs5*vrg5 vrs6*(1-vrg6)];
%************************************************************

% make a matrix of the mean numerical values using subs
realmx = subs(mx,Svr,vr);
% use eigenall.m to get eigenvalues
[lambdas,lambda1,W,w,V,v]= eigenall(realmx);
sensmx = v*w'/(v'*w);   % sensitivities of matrix elements
elastmx = (sensmx.*realmx)/lambda1; % element elasticities
numvrs = length(vr); % how many vital rates?
vrsens = zeros(1,numvrs); % initialize vital rate sens.

% a loop to calculate sensitivity for each vital rate
for xx=1:numvrs
         % derivatives of elements with respect to vital rates
             diffofvr = double(subs(diff(mx,Svr(xx)),Svr,vr)); %
         this was changed: 4/22/04
             % sum up to get vital rate sensitivities

             vrsens(xx) = double(sum(sum(sensmx.*diffofvr))); % this
         was changed: 2/22/2003
         vrsd (xx) = vrvar(xx)^(1/2);
```

```
        VSS (xx) = ((vrsens(xx)*vrsd(xx))/lambda1);

end; % xx

vrelast = ((vrsens.*vr)/lambda1); % calculate elasticities
disp('based on 30 years data')
disp('Matrix element sensitivities and elasticities:')
disp(sensmx); disp(elastmx);
disp('Below are the vital rate results');
disp('vital rates:');
disp(Svr)
disp('sensitivities');
disp(vrsens)
disp('elasticities');
disp(vrelast)
disp('vrsd');
disp(vrsd)
disp('VSS');
disp(VSS)
```

APPENDIX 5

A.5 Different computer programs used test the robustness of model predictions

There are a variety of different programs available for performing PVA, which simulate the future projection of a population, based on a pre-defined set of parameters. These programs may make different assumptions, or may utilise slightly different parameters in performing the analyses. Therefore, for accurate predictions to be made about the future viability of a population, it is vital that the chosen model suits the data available, and that the correct parameters are used. To investigate the robustness of model predictions, four programs were utilised to perform PVA of the European captive population of black rhinoceros; individual-based models Vortex 9.93 (Lacy et al. 2005) and ZooRisk 3.8 (Earnhardt et al. 2008), and projection matrix models either using RAMAS Metapop (Akçakaya 2002), or constructed in MATLAB 2008a (The MathWorks Inc 2008) as previously described (section 3.2.2).

A.5.1 Methods

Model specific details are provided in the following sections. For each program, deterministic and stochastic models were run, and the population was projected for either 10 or 100 years, with single-year time steps in each case. Different projection time-scales were used in order to obtain approximations of future population size in the short and long term, the first being more useful from a current population management perspective, and the second to approximate risk of extinction or population trends over the longer-term for species persistence. Each run of the stochastic model in each program consisted of 1000 iterations, to allow for good representation of parameter combinations and produce a reliable estimate of future population size and growth rate.

A.5.1.1 Vortex

Vortex is an individual-based simulation model, which includes both males and females in the population. The program models population dynamics based on specified probabilities for births and deaths, and tracks the fate of hypothetical individuals into the future. This program is required to make certain assumptions about the data; for example, once individuals reach breeding age, reproduction is assumed to be equal

across all ages. Similarly, pre-reproductive age mortality is entered on a yearly basis, but once individuals enter into the reproductive category, mortality is assumed to be equal across all ages. For each year of the simulation, Vortex simulates environmental variation in the probabilities of reproduction and mortality, by selecting a random number from a binomial distribution, defined by the provided mean and standard deviation in vital rates.

The European captive black rhino population was modelled as a single population and was deemed to be extinct when only one sex remains. Percentage risk of mortality was calculated for males and females, and the percentage of females breeding each year was calculated from females age 5-32. The percentage of males successfully siring offspring was calculated as the number of known sires in a given year divided by the number of males aged 7-32 that could have sired an offspring during that year. Parameters were calculated from the raw data for each year between 1986 and 2010, then averaged across years, and the standard deviation (SD) calculated for the two time periods.

A.5.1.2 ZooRisk

The ZooRisk program uses an individual-based approach, including both males and females in the population. This program provides a quantitative assessment based on a population's history, whilst also taking into account the biology of small populations, and the ability to manage captive populations. The fates of individuals in the population and their hypothetical future offspring are calculated based on values for mortality (male and female), the probability of females breeding, and whether males are of breeding age or not. As opposed to incorporating the observed variance in vital rates, demographic stochasticity is automatically incorporated into the ZooRisk model using Monte Carlo simulations and the binomial distribution to determine the number of deaths in the population, the sex of an offspring, and whether or not a female is pulled into the breeding pool in a given year.

This program has been designed so that the input values for reproduction and mortality can be extracted directly from the species studbook. Although these extracted values were generally similar to the vital rates calculated from raw data, due to the small population size and to make the model comparable to the others used,

these were also manually adjusted using calculations from the raw dataset. In this program, female reproduction is based on probability of breeding (total offspring) rather than fecundity (same sexed offspring only), so calculated values for fecundity were doubled. Additionally, model parameters such as breeding group composition and number of years between pairings were inputted based on different management scenarios; the number of females per breeding group was either one or two, based on the two management scenarios currently in use at European institutions. Additionally, the birth sex ratio (BSR) of the EEP population during the 25 year period between 1st January 1986 and 31st December 2010 was significantly different from parity (BSR=0.3853, χ^2=5.7339, df=1, P=0.017 based on 42 male and 67 female births). This ratio reflects a greater number of females calves born into this population over this time-frame, and this ratio was taken into account in simulated projections. Although the BSR was not significantly different from parity during the latter 10 year period, (χ^2=3.1026, df=1, P0.078 based on 14 male and 25 female births), a scenario of BRS 0.4 was also used.

A.5.1.3 RAMAS Metapop

The matrix-based software package RAMAS Metapop was used to create a female-based transition matrix containing age-specific survival and fecundity, similar to the model described in section 3.2.4.1, to model population growth. Using the calculated vital rates of fecundity and mortality, a transition matrix was constructed containing the contribution of each individual to each stage at the subsequent time step. This contribution can either be through surviving and remaining in the same age-class, surviving and progressing to the next age-class, or through reproduction. These matrix elements were calculated for each year of the two data extraction windows, and the standard deviation in matrix elements calculated between years. For each year of the simulation, environmental stochasticity in reproduction and survival was modelled using a lognormal distribution, defined by the specified mean and standard deviation.

A.5.2 Results

Table A.5.1: Output from MATLAB model, based on two time periods for data collection 1) 1st January 1986 to 31st December 2010 and 2) 1st January 2001 to 31st December 2010. Table includes deterministic growth rate (det. λ), mean and standard deviation (SD) of stochastic growth rate (stoc. λ) and mean and SD in final number of females in the population after either 10 or 100 years simulated projection.

Time period	Projection period	Det. λ	Mean Stoc. λ	SD Stoc. λ	Starting N (F only)	Mean final N (F only)	SD final N (F only)
1	10 years	1.0211	1.0270	0.0134	51	67.09	8.81
	100 years		1.0212	0.0048		463.21	237.14
2	10 years	1.0012	1.0156	0.0086	51	59.72	5.05
	100 years		1.0052	0.0033		89.87	29.89

Table A.5.2: Output from Vortex model, based on two time periods for data collection 1) 1st January 1986 to 31st December 2010 and 2) 1st January 2001 to 31st December 2010. Table includes deterministic growth rate (det. λ), mean and standard deviation (SD) of stochastic growth rate (stoc. λ) and mean and SD in final population size after either 10 or 100 years simulated projection.

Time period	Projection period	Det. λ	Mean Stoc. λ	SD Stoc. λ	Starting N (M&F)	Mean final N (M&F)	SD final N (M&F)
1	10 years	1.0220	1.0356	0.0620	73	93.95	8.77
	100 years		1.0222	0.0550		802.10	486.74
2	10 years	1.0050	1.0222	0.0450	73	89.65	9.05
	100 years		1.0060	0.0440		144.87	73.97

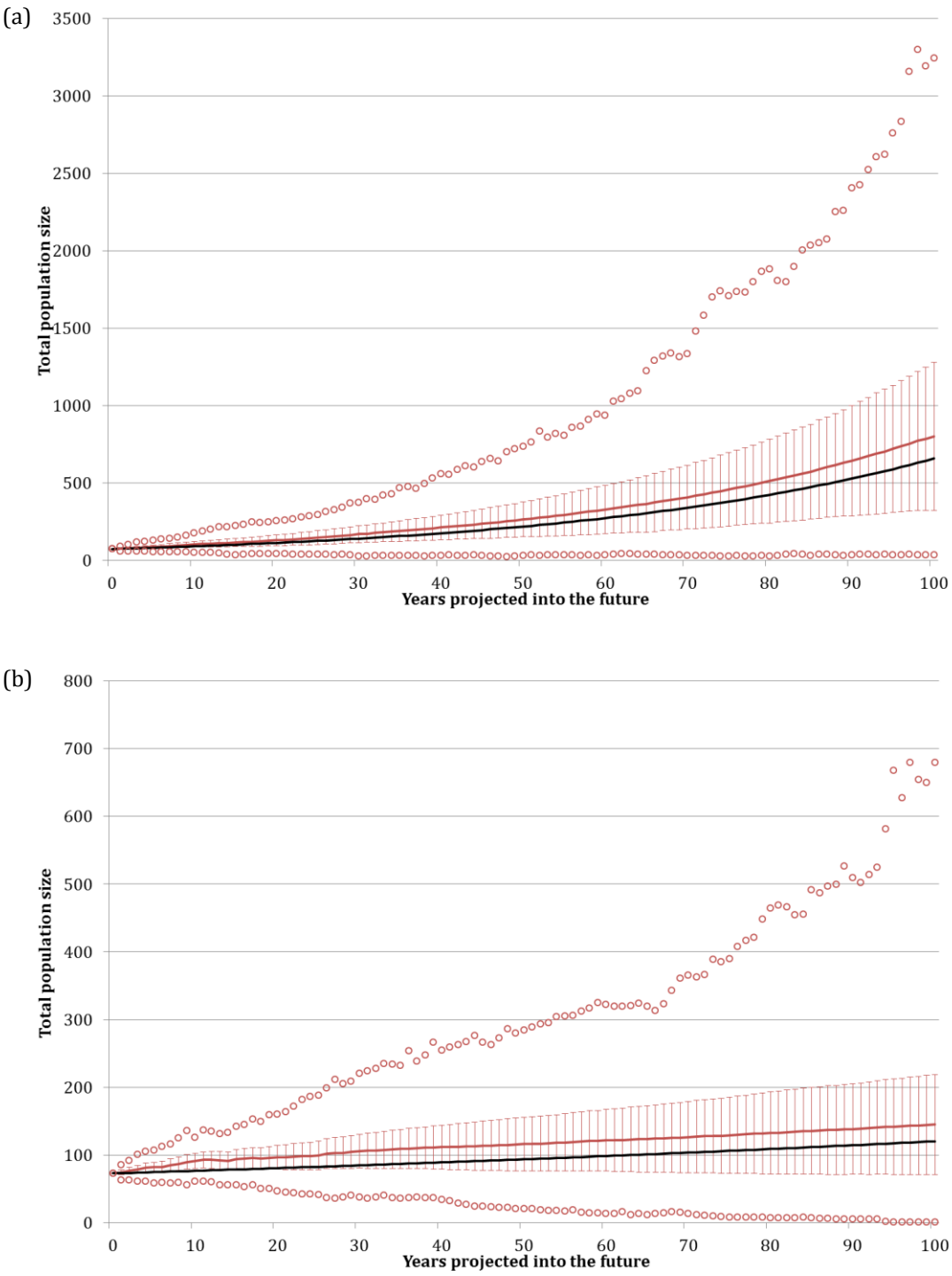


Figure A.5.1: Population size (males and females combined) projection from Vortex model showing results from simulations based on the last a) 25 and b) 10 year data. The average projected population size based on stochastic simulations is represented by the red line; error bars represent one standard deviation in population size across 1000 iterations; red markers represent the minimum and maximum population size estimates obtained from simulations for each year of projection. The black line represents the deterministic population projection, which is the projected growth of the population under a constant environment. Note the difference in scale on the y-axis between the two scenarios, representing the difference in final population size by 100 years.

Table A.5.3: Output from ZooRisk model, based on two time periods for data collection 1) 1st January 1986 to 31st December 2010 and 2) 1st January 2001 to 31st December 2010. Table includes deterministic growth rate (det. λ), mean and standard deviation (SD) of stochastic growth rate (stoc. λ) and mean and SD in final population size after either 10 or 100 years simulated projection.

Time period	Breeding group	Birth Sex Ratio	Projection period	Det. λ	Mean Stoc. λ	SD Stoc. λ	Starting N (M&F)	Mean final N (M&F)	SD final N (M&F)
1	1.1	0.5	10 years	1.0110	1.0109	0.0082	78	87.39	7.28
	1.1	0.5	100 years	1.0146	1.0138	0.0042		323.10	117.66
2	1.1	0.5	10 years	1.0038	1.0044	0.0079	78	81.42	6.50
	1.1	0.5	100 years	1.0053	1.0022	0.0043		110.00	42.66
1	1.2	0.5	10 years	1.0110	1.0114	0.0081	78	87.20	7.13
	1.2	0.5	100 years	1.0306	1.0302	0.0025		1550.35	367.42
2	1.2	0.5	10 years	1.0272	1.0262	0.0081	78	100.80	7.82
	1.2	0.5	100 years	1.0144	1.0136	0.0030		321.51	91.72
1	1.1	0.3853	10 years	1.0110	1.0110	0.0081	78	87.80	6.79
	1.1	0.3853	100 years	1.0021	1.0011	0.0066		107.61	56.28
2	1.1	0.4	10 years	1.0038	1.0040	0.0082	78	80.96	6.47
	1.1	0.4	100 years	0.9961	0.9946	0.0068		56.31	29.39
1	1.2	0.3853	10 years	1.0405	1.0380	0.0085	78	113.70	9.06
	1.2	0.3853	100 years	1.0424	1.0394	0.0023		3759.40	761.46
2	1.2	0.4	10 years	1.0272	1.0257	0.0083	78	101.08	8.10
	1.2	0.4	100 years	1.0223	1.0210	0.0026		636.73	150.50

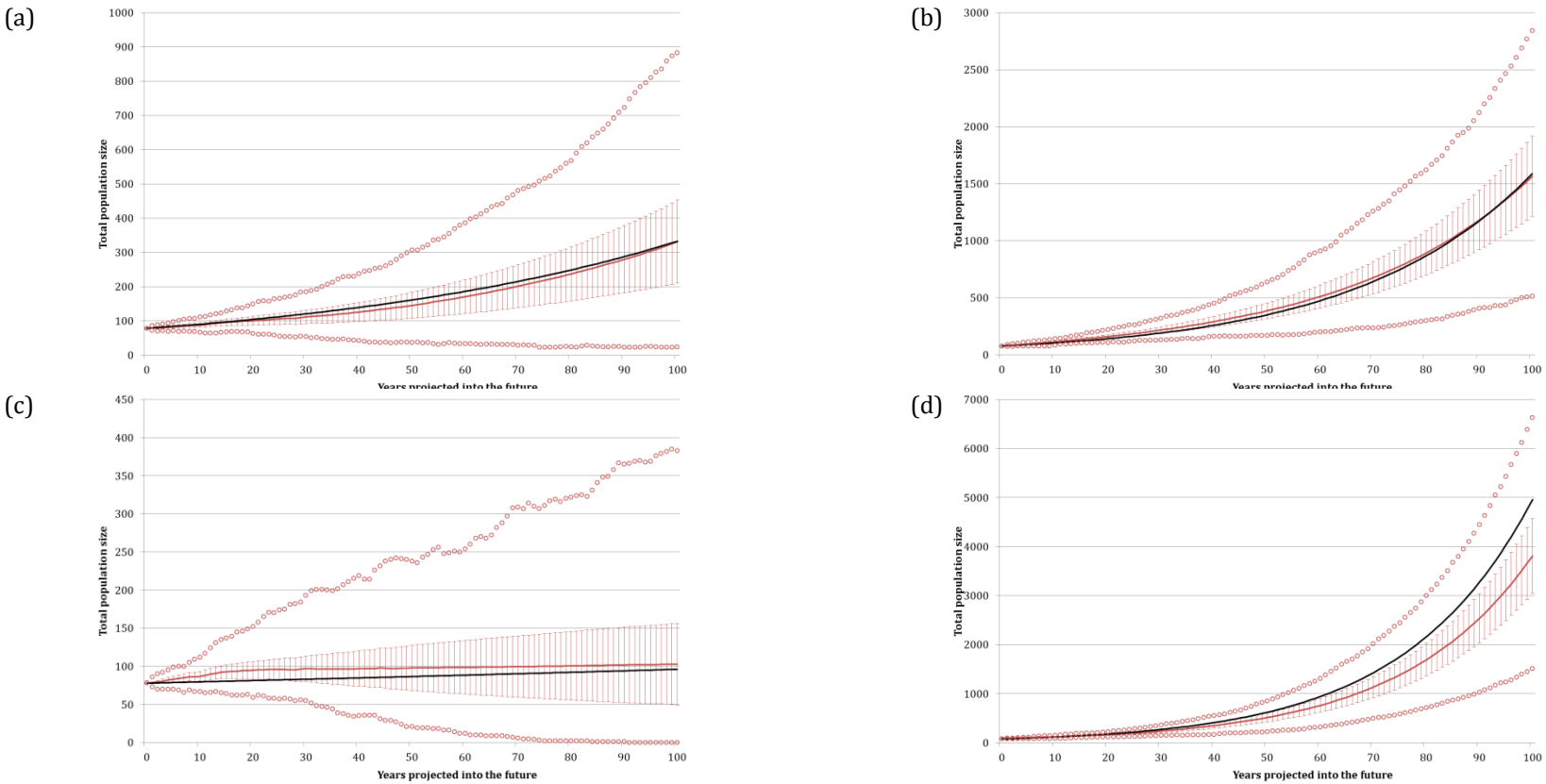


Figure A.5.2: Population size (males and females combined) projection from ZooRisk model showing results from simulations based on the last 25 year data, and based on (a)breeding group composition 1.1, and BSR 0.5, (b) breeding group composition 1.2, and BSR 0.5, (c) breeding group composition 1.1, and BSR 0.3853, or (d) breeding group composition 1.2, and BSR 0.3853. The average projected population size based on stochastic simulations is represented by the red line; error bars represent one standard deviation in population size across 1000 iterations; red markers represent the minimum and maximum population size estimates obtained from simulations for each year of projection. The black line represents the deterministic population projection, which is the projected growth of the population under a constant environment. Note the difference in scale on the y-axis between the two scenarios, representing the difference in final population size by 100 years.

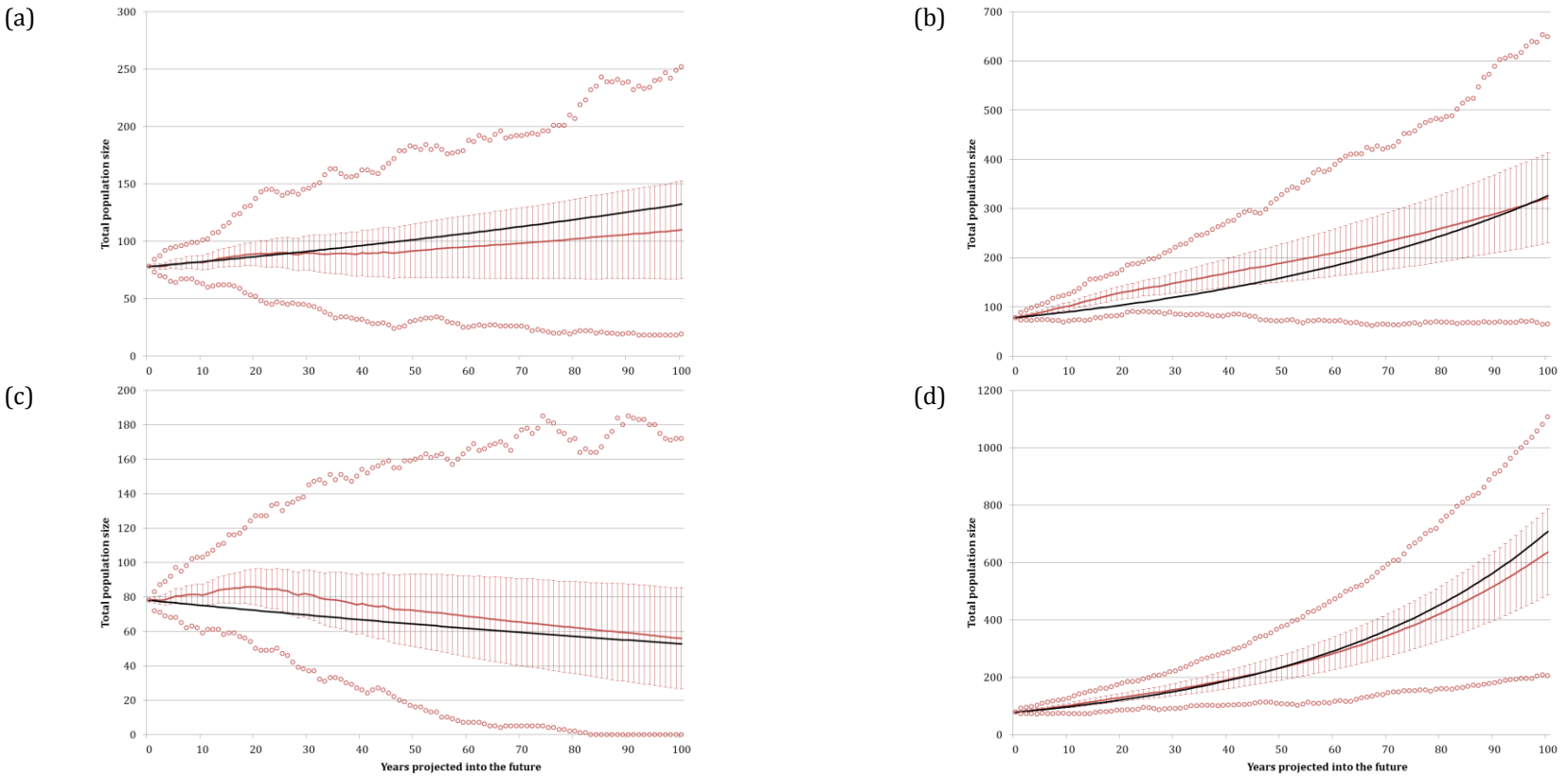


Figure A.5.3: Population size (males and females combined) projection from ZooRisk model showing results from simulations based on the last 10 year data, and based on (a)breeding group composition 1.1, and BSR 0.5, (b) breeding group composition 1.2, and BSR 0.5, (c) breeding group composition 1.1, and BSR 0.4, or (d) breeding group composition 1.2, and BSR 0.4. The average projected population size based on stochastic simulations is represented by the red line; error bars represent one standard deviation in population size across 1000 iterations; red markers represent the minimum and maximum population size estimates obtained from simulations for each year of projection. The black line represents the deterministic population projection, which is the projected growth of the population under a constant environment. Note the difference in scale on the y-axis between the two scenarios, representing the difference in final population size by 100 years.

Table A.5.4: Output from RAMAS Metapop model, based on two time periods for data collection 1) 1st January 1986 to 31st December 2010 and 2) 1st January 2001 to 31st December 2010. Table includes deterministic growth rate (det. λ), mean and standard deviation (SD) of stochastic growth rate (stoc. λ) and mean and SD in final number of females in the population after either 10 or 100 years simulated projection.

Time period	Projection period	Det. λ	Mean Stoc. λ	SD Stoc. λ	Starting N (F only)	Mean final N (F only)	SD final N (F only)
1	10 years	1.0205	1.0314	0.0059	51	69.21	18.10
	100 years		1.0234	0.0045		514.17	524.50
2	10 years	1.0015	1.0156	0.0025	51	59.51	13.20
	100 years		1.0035	0.0054		72.56	77.00

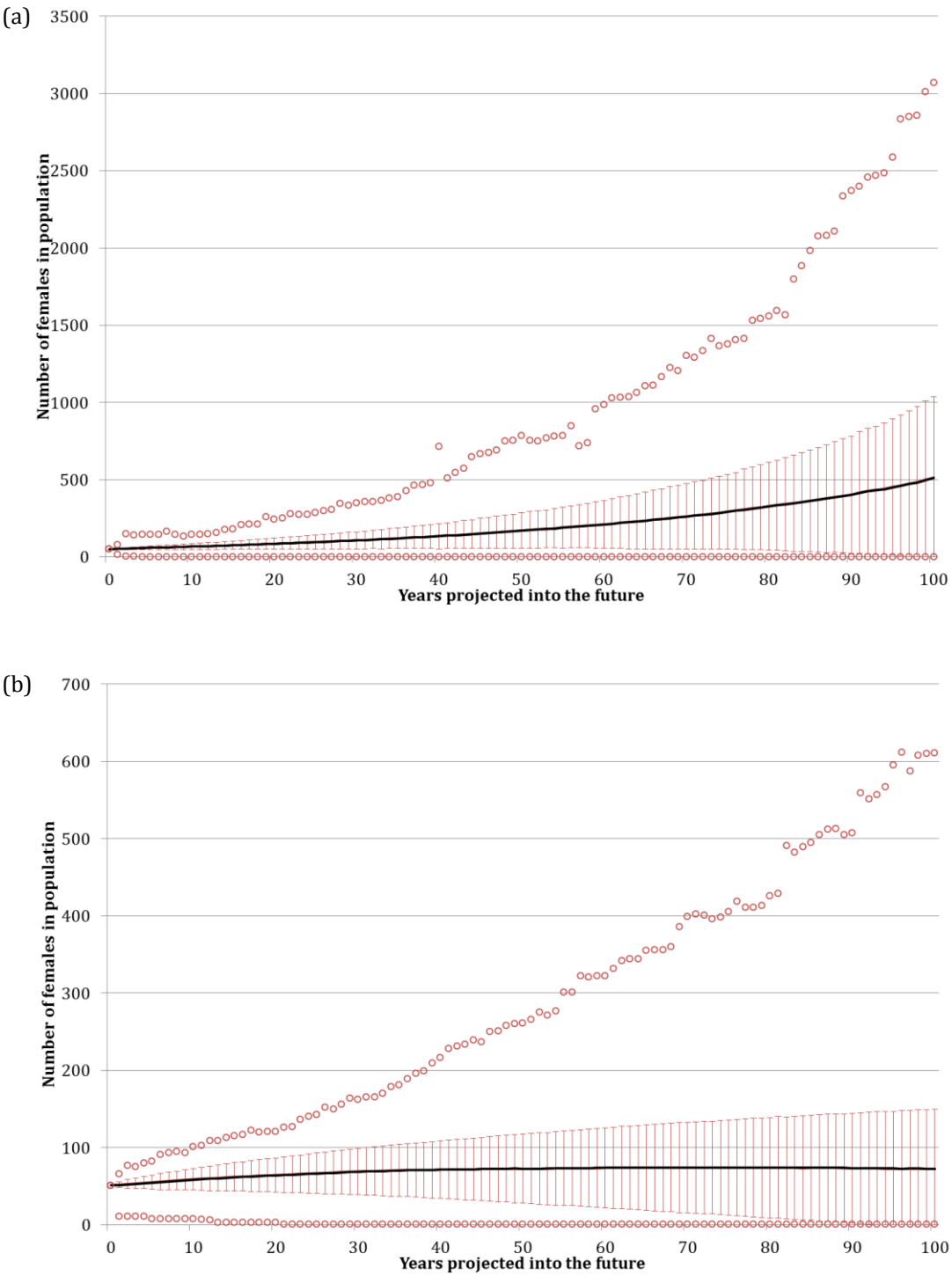


Figure A.5.4: Population size (females only) projection from RAMAS Metapop model showing results from simulations based on the last a) 25 and b) 10 year data. The average projected population size based on stochastic simulations is represented by the red line; error bars represent one standard deviation in population size across 1000 iterations; red markers represent the minimum and maximum population size estimates obtained from simulations for each year of projection. The black line represents the deterministic population projection, which is the projected growth of the population under a constant environment. Note the difference in scale on the y-axis between the two scenarios, representing the difference in final population size by 100 years.

APPENDIX 6

A.6 Analyses of behavioural, environmental and social variables and reproductive categories

Table A.6.1: Analyses of individual rhino behaviour across reproductive categories. Variables were analysed either using Mann Whitney U (2 groups of breeding status) or Kruskal Wallis (3 groups of bred within 7 years).

	Males				Females			
	Breeding status [a]		Bred within 7 years [b]		Breeding status [a]		Bred within 7 years [b]	
	Mann-Whitney U	P	Kruskal Wallis	P	Mann-Whitney U	P	Kruskal Wallis	P
Pacing	28.5	0.875	0.043	0.979	90.0	0.377	1.219	0.544
Charging	28.5	0.875	1.030	0.598	123.0	0.667	2.270	0.321
Behaviour towards keepers:								
Approach keepers	24.0	0.562	1.429	0.490	91.0	0.400	0.971	0.615
Seek contact from keepers	30.5	1.000	0.087	0.957	108.0	0.886	0.097	0.953
Interested in keeper activity	32.5	0.792	0.376	0.829	95.0	0.498	1.427	0.490
Nervous around keepers	31.0	0.689	1.098	0.577	93.5	0.621	0.451	0.798
Approach new people	22.0	0.428	0.859	0.651	103.5	0.728	1.426	0.490
Aggressive towards people	21.5	0.368	1.047	0.592	91.5	0.400	0.787	0.675
Behaviour towards environment:								
Watchful of surroundings	21.5	0.368	1.003	0.606	74.0	0.120	5.064	***0.08***
Curious of new objects/surroundings	15.0	0.118	3.024	0.220	106.0	0.822	0.491	0.782
Approach novel objects/surroundings	33.0	0.792	2.250	0.325	114.5	0.918	0.014	0.993
Nervous within environment	26.5	0.713	0.199	0.905	72.0	0.101	3.997	0.137
Response to unexpected events	25.5	0.864	3.020	0.221	103.5	0.728	1.808	0.405
Active/explore environment	38.0	0.428	0.900	0.638	84.0	0.257	1.617	0.446
Startled by sudden sounds/movements	26.0	1.000	2.088	0.352	86.0	0.664	0.905	0.636
Changeable	26.0	0.713	1.008	0.604	71.0	0.093	4.827	0.090

[a] Breeding status – proven breeder (has produced a live calf during lifetime) vs. non-proven breeder (has never produced a live calf). [b] Bred within the last 7 years – proven breeder that has bred during the last seven years vs. proven breeder but has not produced a calf during the last seven years vs. non-proven breeder. P values in bold represent those significant at the 0.05 level, tendencies (P<0.10) are denoted by bold italics.

Table A.6.2: Analyses of environmental factors across reproductive categories. Variables were analysed either using cross-tabulation & Pearson X^2 (to compare the proportion of individuals P vs. NP or P<7, P>7 or NP in each category), or using either Mann Whitney U (2 groups of breeding status; grey cells) or Kruskal Wallis (3 groups of bred within 7 years; grey cells).

	Males				**Females**			
	Breeding status [a]		**Bred within 7 years [b]**		**Breeding status [a]**		**Bred within 7 years [b]**	
	Pearson X^2	**P**	**Pearson X^2**	**P**	**Pearson X^2**	**P**	**Pearson X^2**	**P**
Enclosure boundary:								
Solid walls included in enclosure boundary (*yes vs. no*)	3.484	***0.062***	4.444	0.108	1.050	0.306	3.246	0.197
% Solid walls in enclosure boundary	4.0	0.400	4.625	***0.099***	40.5	1.000	5.409	***0.067***
% Fence in enclosure boundary	8.5	0.315	2.705	0.259	53.5	0.678	4.316	0.116
Enclosure area (m^2):	28.5	0.875	0.466	0.792	122.0	0.697	1.085	0.581
Visitor access:								
% Perimeter of outdoor enclosure has visitor access	38.0	0.428	0.875	0.646	93.0	0.448	1.625	0.444
On-show vs. off-show	1.371	0.242	2.286	0.319	0.536	0.464	0.952	0.621
Visitor access to indoor enclosures (*yes vs. no*)	0.027	0.869	1.504	0.471	0.368	0.544	5.162	***0.076***
Opportunity to escape from view (*yes vs. no*)	0.356	0.551	0.889	0.641	0.536	0.464	0.952	0.621

[a] Breeding status – proven breeder (has produced a live calf during lifetime) vs. non-proven breeder (has never produced a live calf). [b] Bred within the last 7 years – proven breeder that has bred during the last seven years vs. proven breeder but has not produced a calf during the last seven years vs. non-proven breeder. P values in bold represent those significant at the 0.05 level, tendencies (P<0.10) are denoted by bold italics.

Table A.6.3: Analyses of social factors and reproductive status. Variables were analysed either using cross-tabulation & Pearson X^2 (to compare the proportion of individuals P vs. NP or P<7, P>7 or NP in each category), or using either Mann Whitney U (2 groups of breeding status; grey cells) or Kruskal Wallis (3 groups of bred within 7 years; grey cells).

	Males				**Females**			
	Breeding status [a]		**Bred within 7 years [b]**		**Breeding status [a]**		**Bred within 7 years [b]**	
	Pearson X^2	**P**	**Pearson X^2**	**P**	**Pearson X^2**	**P**	**Pearson X^2**	**P**
No. rhinos at institution:								
Males	1.067	0.608	1.167	0.558	3.453	***0.081***	3.520	0.172
Females	1.067	0.608	1.167	0.558	3.453	***0.081***	3.520	0.172
Housed with other rhinos:								
Housed with same sex *Not at all; Some of the time; All of the time*	3.810	***0.051***	3.810	0.149	3.151	0.207	3.987	0.408
Housed with opposite sex *Not at all; Some of the time, not limited to oestrus; During oestrus only; All the time*	1.920	0.589	6.200	0.401	2.946	0.400	4.571	0.600
Housed near other rhinos:								
Housed near same sex (*yes vs. no*)	0.950	0.330	2.812	0.245	0.238	0.626	1.905	0.386
Housed near opposite sex (*yes vs. no*)	3.810	***0.051***	3.810	0.149	0.536	0.464	1.693	0.429

[a] Breeding status – proven breeder (has produced a live calf during lifetime) vs. non-proven breeder (has never produced a live calf). [b] Bred within the last 7 years – proven breeder that has bred during the last seven years vs. proven breeder but has not produced a calf during the last seven years vs. non-proven breeder. P values in bold represent those significant at the 0.05 level, tendencies (P<0.10) are denoted by bold italics.

APPENDIX 7

A.7 Published papers in support of this thesis

1. Watson, R., Munro, C., Edwards, K.L., Norton, V., Brown, J.L., Walker, S.L., 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. http://dx.doi.org/10.1016/j.ygcen.2013.02.001