Reproductive physiology in the wild white rhinoceros (*Ceratotherium simum*): New insights for enhanced breeding success

by

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Thesis.

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I dedicate this thesis to Mariga, Pula,
Mohobe and Kamogelo, who were brutally killed by
poachers in Lapalala Wilderness during the timeframe
of this doctorate. And to Mohlaki. You are the stars.
And my inspiration.

Summary

The African white rhinoceros (*Ceratotherium simum*), having been rescued from extinction at the end of the 19th century, is one of the five remaining species of rhinoceros, along with the African black (*Diceros bicornis*), Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran (*Dicerorhinus sumatrensis*) species. The population of *C. simum* faces an uncertain future, primarily because of the extremely high demand for its horn, which is being used illegally as an ingredient of traditional Asian medicine and in the manufacture of ceremonial curved daggers in the Middle East. Rhinoceros horn is also regarded as a status symbol for Chinese and Vietnamese elites. Breeding in captive and semi-captive environments could play a critical role in the survival of this conservation-dependent species, because captive populations can serve as genetic reservoirs and sources of animals for reintroduction into the wild. However, captive white rhinoceros females in captivity, in contrast with their wild counterparts, reproduce poorly and thus show a negative population growth rate (–3.5% pa for the entire captive population). For this reason, the sustainability of the captive population is in jeopardy. The causes of impaired reproduction are poorly understood. Endocrine monitoring of the ovarian activity in captive females has revealed acyclic periods and wide variation in cycle length, both believed to have a pathological origin. On the other hand, it is not known whether wild rhinoceros females show similar characteristics – we do not have a solid foundation of the normal reproductive biology of the species, and the studies to remedy this situation are a main aspect of this thesis.

The literature review introduces the current problems seen in breeding white rhinoceros in facilities worldwide, highlighting potential negative consequences for the survival of the species and analyzes variations in research findings. This allowed the prioritization of problems and the formulation of hypotheses. The first experimental chapter describes the development of a non-invasive method for collecting endocrine data on a regular basis from free-living white rhinoceros females and provides the first data using this method. Fresh fecal samples were collected at least once a week for 12 months from six adult females at Lapalala Wilderness and stored frozen at -20°C until analysis with an enzyme immunoassay utilising an antiserum raised against 5α-pregnan-3β-ol-20-one which cross reacts with a number of progestagens. The outcome was long-term profiles of fecal progestagen metabolite (fPM) for individual females in the wild, providing information on pregnancy status, and showing how the technique can be used for detection of pregnancy without the need to immobilize and/or relocate the animal. This information can be used to optimize breeding management of wild populations. The second experimental chapter investigates the estrous cycle of females in the wild and tests 3 hypotheses: 1) estrous cycles can be described in the wild using non-invasive techniques; 2) females in the wild only show one type of estrous cycle; 3) females in the wild show fewer periods of acyclicity than females in captivity. By measuring the fPM concentrations for 20 months in fresh fecal samples from 5 adult, reproductively successful, free-living females, we show that the estrous cycle in the wild lasts for 30.6 ± 7.7 days (n = 7). Periods of acyclicity were detected and appeared to be related to the

presence of a new adult bull. We conclude that the irregular cyclicity reported for captive animals is not normal and could explain their poor fertility. The data are limited but suggest that white rhinoceros females in the wild might need external stimuli to ovulate.

The final experimental chapter focuses on the development of a protocol for synchronization of estrus and ovulation in captive females, with a view to offering timed natural breeding and assisted reproduction. Previous publications have only considered retrospective analysis of case studies, but we attempted a controlled experiment with 3 female southern white rhinoceroses. The treatment protocol, based on protocols used for the mare and informed by previous attempts in other species of rhinoceros, utilized a synthetic progestagen treatment followed by a slow-release GnRH analogue. Success was determined by recording fPM concentrations in daily fecal samples for 6 months before and during treatment, along with observations of physical signs of estrus. Luteal activity was seen in all three females within the sample collection period. However, these observations did not seem to be related to the treatment protocol. Potential explanations include inaccuracy in assay results, so an alternative assay is being investigated. Improvement of the protocol is essential if we are to induce ovulation reliably and thus assist breeding in the white rhinoceros. A coincidental finding was a spontaneous synchronized luteal phase in two females that were housed together, which has not been reported before in the literature. Collectively, the studies described in this thesis present a new approach toward understanding and solving the breeding problem

seen in the white rhinoceros in captivity. The development of an efficient, non-invasive method for monitoring estrous cycles and pregnancy in wild females has revealed major aspects of normal reproductive biology, the first step towards analysis of reproductive problems in captive females. This work has highlighted critical physiological similarities and differences between captive and wild individuals. Finally, we have made a first step towards the development of a functional protocol for inducing estrus in captive females. The methods and techniques present possible avenues for future reproductive research aiming to improve breeding management strategies of wild and captive populations for maximum breeding output.

Key words: Progestagen, ovarian cyclicity, reproduction, wild populations, non-invasive hormone measurement, *Ceratotherium simum*

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Statement of candidate contribution

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DECLARATION FOR THESES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION

This thesis contains published work and work prepared for publication, **some of which has been co-authored**. The bibliographical details of the work and where it appears in the thesis are outlined below.

Research papers

Goot van der, A.C., Dalerum, F., Ganswindt, A., Martin, G.B., Millar, R.P., Paris, M.C.J., 2013. Faecal Progestagen Profiles in Wild Southern White Rhinoceros (*Ceratotherium simum simum*). Afr. Zool. 48, 143-151.

A slightly modified version of this research paper is presented as Chapter Three of this thesis. I developed the experimental design and undertook most of the fieldwork in this paper, receiving assistance from volunteering students. I conducted all data analysis with guidance from Andre Ganswindt, Stefanie Ganswindt and Fredrik Dalerum. I wrote the manuscript, which was subsequently edited by Monique Paris, Fredrik Dalerum, Andre Ganswindt, Graeme Martin and Robert Millar.

Goot van der, A.C., Martin, G.B., Millar, R.P., Paris, M.C.J., Ganswindt, A., 2015. Profiling patterns of fecal 20-oxopregnane concentrations during ovarian cycles in free-ranging southern white rhinoceros (*Ceratotherium simum simum*). Animal Reproduction Science, 161, 89-95.

A slightly modified version of this research paper is presented as Chapter Four of this thesis. I undertook the fieldwork in this paper, receiving assistance from volunteering students. I conducted most data analysis with guidance from Andre Ganswindt and Stefanie Ganswindt. I wrote the manuscript, which was subsequently edited by Andre Ganswindt, Graeme Martin, Robert Millar and Monique Paris.

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Chapter 1

General introduction

1.1. Historical background

The African white rhinoceros (*Ceratotherium simum,* Perissodactylae: Rhinocerotidae), which has been rescued from extinction at the end of the 19th century, is one of the five species of rhinoceroses extant today. With a bodyweight up to an impressive 3 tonnes it is the second most massive land mammal on earth and carries high biological, economic, cultural and recreational values [Owen-Smith, 1992; Emslie et al., 2009]. Together with the other surviving rhinoceros species: African black (*Diceros bicornis*), Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus)* and Sumatran (*Dicerorhinus sumatrensis*) [Owen-Smith, 1992], the white rhinoceros population is looking at an uncertain future. One of the major causes of decline is the demand for its horn, which is being used as an ingredient in traditional medicine and also in the making of ceremonial curved daggers in the Middle East [Owen-Smith, 1992; Emslie et al., 2009]. Horn poaching involves killing the animal and cutting off its horns. The demand for rhinoceros horn in the world is increasing and finding strategies to fight against the poaching plays a key role in maintaining the rhinoceros populations today. Due to illegal hunting, the black rhinoceros population, for example, has faced a population decline of 96% between 1970 and 1992 [Emslie et al., 2009].

Two genetically distinct subspecies of white rhinoceros exist, namely the northern white rhinoceros (*Ceratotherium simum cottoni*) and the southern white rhinoceros (*Ceratotherium simum simum*) [Owen-Smith, 1992]. Only 5 individuals remain of the northern subspecies and following ground surveys in 2009 that did not find any sign of remaining individuals in the wild [Emslie et al., 2009], a number of captive individuals have been re-introduced into a secure reserve in Kenya to increase breeding success [Milliken et al., 2009]. The southern white rhinoceros population is currently estimated to be 20,429 of which 93% live in South Africa [Emslie and Knight, 2014]. Although this number seems viable, rapid decimation of the population, as observed in other rhinoceros species, is possible and should be avoided. Being classified as “Near Threatened” on the IUCN’s Red List of Threatened Species, the southern white rhinoceros population is dependent on effective protection and intensive conservation and management [Amin et al., 2003; Hermes et al., 2005]. Currently, illegal hunting in South Africa is steadily increasing with more than 1100 rhinoceroses poached in 2013, while a total of 210 were illegally killed between 2006 and 2009 [Milliken et al., 2009; Brooks, 2011; Emslie and Knight, 2014]. The population depends largely on governmental support by a single country, i.e. South Africa and we should be readily aware of potential political changes influencing white rhinoceros protection efforts. When we meet a point where the population becomes vulnerable again – as observed a century ago with only approximately 200 individuals remaining in the wild [Rookmaaker, 2001] – successful breeding strategies become indispensable in order to preserve the species.

1.2. Motivation

Ex situ breeding plays a key role in maintaining the rhinoceros populations today, with these captive populations serving as potential reservoirs for reintroduction into the wild. However, white rhinoceros females in captivity, especially within the captive-born (F1) generation, show poor reproductive health and aberrant hormonal cycling patterns [Swaisgood et al., 2006]. We do not see this in other rhinoceros species [Roth, 2006]. With an alarmingly low birth rate in captivity (-3.5% as a percentage of the entire population), analysts predict a crisis in the coming years [AZA, 2014; Swaisgood et al., 2006; Roth, 2006]. Currently, the worldwide captive white rhinoceros population (n = 747) is stationary ($\lambda = 1.001$). However, this includes the continuous importation of new individuals from the wild [Foose et al., 2006]. This means that the captive population is not self-sustainable.

In contrast, behavioral studies suggest that southern white rhinoceroses in the wild do not show reproductive impairment [Owen-Smith, 1992; Swaisgood et al., 2006]. Birth rates of 8-10% are being reported for free-living populations [Emslie et al. 1999]. This observed sustainable rate of natural reproduction is an essential requisite for making intensive research highly worth its effort. Our understanding of the reproductive biology of the southern white rhinoceros is still limited and details are largely unknown. Only a few observations have been made related to female reproduction in the wild [Skinner and Smithers, 1990; Owen-Smith, 1992; Bertschinger, 1994] and to

date, no endocrine data are available on cyclicity and pregnancy in free-living white rhinoceroses.

Hormones are often measured in blood samples but collecting regular blood samples from free-living rhinoceroses is virtually impossible. In the wild a rhinoceros needs to be sedated, captured and intensively monitored by an anaesthetic team for a single blood sample from either ear or leg vein and most females have a young at foot, so the young one also needs to be darted. With the development of fecal hormone assays, it is now possible to collect samples in a non-invasive way. Apart from the practical advantages, this technique also bypasses the potential negative effects of stress on the results when using invasive methods [Christensen et al., 2006; Wittemyer et al., 2007]. Investigating reproductive profiles and correlating potential influencing factors will increase our knowledge of the reproductive physiology of the female white rhinoceros in its natural environment, which can facilitate in understanding existing reproductive health impairment in captivity. It is now possible to quantify progesterone levels non-invasively by measuring 5α-pregnane metabolites in fecal samples of rhinoceroses [Schwarzenberger, 1998]; however, a hormone assay test for measuring fecal estrogen metabolites has not yet been validated for white rhinoceros species [Hindle, 1992; Brown, 2001; Roth, 2006].

Enhancing breeding programmes in captivity by integrating assisted reproduction technologies, where induced ovulation is followed by artificial insemination, is a developing area in zoo- and wildlife management [Hildebrandt et al., 2003]. It can be a vehicle for rapid dispersal of valuable genes and genetic

advancement in order to keep populations healthy, by preventing inbreeding depression through loss of individual reproductive fitness. There have been three artificial inseminations with successful outcome in white rhinoceroses [Hermes, 2009]. However, many attempts have failed and inaccurate detection of estrus in rhinoceroses and poor and inconsistent outcomes of the protocols used to induce the onset of estrus and/or ovulation are experienced throughout the captive breeding programs [Schwarzenberger et al., 1998; Hermes et al., 2007]. The general lack of knowledge of estrus cycle characteristics in the white rhinoceros contributes to this directly [Hermes et al., 2006]. The full potential of white rhinoceros breeding programs can only be achieved by understanding the proximate mechanisms that regulate reproduction and how these are modified by extrinsic and intrinsic factors. The general hypothesis of this thesis is that better understanding basic endocrine reproductive events in free-living female white rhinoceroses, including estrous cycle length and environmental influences on reproduction, will allow the development of far more successful protocols for reproductive management in the wild and in captivity. This information can then be further integrated into research focused on the development of estrus induction protocols.

1.3. Objectives

The main objectives of this study are:

1. To develop and validate an accurate and meaningful method to measure progestagen metabolites non-invasively in the white rhinoceros;

2. To provide longitudinal data on fecal progestagen metabolite (FPM) concentrations in pregnant and non-pregnant wild individuals;
3. To determine estrous cycles in wild white rhinoceros females using non-invasive monitoring techniques;
4. To determine whether females in the wild only show one type of cycle;
5. To determine if females in the wild show limited periods of acyclicity compared to females in captivity; and
6. To identify the treatment schedule that provides the highest estrus response and best induction of estrus in the white rhinoceros.

1.4. Thesis structure

The outline of this thesis was structured in a way that will enable the reader to follow the line of thought from one chapter into the next. This thesis begins with a literature review in Chapter 2. Chapter 3 focuses on the development of a non-invasive method to collect endocrine data on a regular basis from free-living white rhinoceros females. Chapter 4 examines estrous cycle characteristics in wild white rhinoceros females. Finally, Chapter 5 aims to develop a protocol for synchronization of estrus and ovulation in captive white rhinoceros females, with the objective of using the acquired information about the normal reproductive cycle in Chapters 3 and 4 for a more accurate approach. The general discussion of the thesis is presented in Chapter 6.

1.5. References

Amin, R., M. Bramer, and R. Emslie (2003), intelligent data analysis for conservation: experiments with rhino horn fingerprint identification, *Knowledge-Based Systems, 16*(5-6), 329-336.

AZA, 2014. Rhino Advisory Group's 2014 Research Masterplan. American Zoo and Aquarium Association.

Bertschinger, H. J. (1994), Reproduction in black and white rhinos: a review, *Proceedings of a symposium on rhinos as game ranch animals.* Onderstepoort, Republic of South Africa, 9-10 September 1994, 96-99.

Brooks, M. (2011), African Rhino Specialist Group report/Rapport du Groupe Spécialiste des Rhinos d'Afrique, *Pachyderm, 48,* 8-15.

Brown, J. L., A. C. Bellem, M. Fouraker, D. E. Wildt, and T. L. Roth (2001), Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring, *Zoo Biol., 20*(6), 463-486.

Carlstead, K. and J. L. Brown (2005), Relationships between patterns of Fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros, Zoo Biol., 24(3), 215-232.

Christensen, B. W., M. Troedsson, R. Little, M. Oliva, and L. Penfold (2006), Invasive and non-invasive methods of hormone monitoring in captive wildlife, Rhino Keeper Workshop 2006 Proceedings of the Fourth Rhino Keepers workshop 2005 at Columbus, Ohio. Columbus, Rhino Keepers Workshop 1.

Emslie, R. H., R. Amin, and R. Kock (2009), Guidelines for the in situ re-introduction and translocation of African and Asian

rhinoceros, *Occasional Paper of the IUCN Species Survival Commission, 39*, 16.

Emslie, R. H. and M. H. Knight (2014), Update on African rhino status and trends: from IUCN SSC African Rhino Specialist Group (AfRSG), in Report to CITES Standing Committee 65th meeting, 1-6.

Foose, T. J., and R. J. Wiese (2006), Population management of rhinoceros in captivity. *International Zoo Yearbook 40*, 174-196.

Hermes, R., T. B. Hildebrandt, S. Blottner, C. Walzer, S. Silinski, M. L. Patton, G. Wibbelt, F. Schwarzenberger, and F. Göritz (2005), Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation, *Theriogenology, 63*(1), 219-238.

Hermes, R., et al (2006), The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*), *Theriogenology, 65*(8), 1492-1515.

Hermes, R., F. Göritz, W. Streich, and T. Hildebrandt (2007), Assisted Reproduction in Female Rhinoceros and Elephants – Current Status and Future Perspective, Reproduction in Domestic Animals, 42(s2), 33-44.

Hermes, R. et al. (2009), First successful artificial insemination with frozen-thawed semen in rhinoceros. *Theriogenology* 71, 393.

Hildebrandt, T. B., J. L. Brown, R. Hermes, and F. Göritz (2003). Ultrasound for analysis of reproductive function in wildlife species. In 'Reproductive Science and Integrated Conservation'. (Eds W. V. Holt, A. R. Pickard, X. C. Rodger and D. E. Wildt), 166-182. Cambridge University Press.

Hindle, J. E., E. Most, and J. K. Hodges (1992), Measurement of urinary oestrogens and 20{alpha}-dihydroprogesterone during

ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses, *J.Reprod.Fertil., 94*(1), 237-249.

Kretzschmar, P., U. Gansloßer, and M. Dehnhard (2004), Relationship between androgens, environmental factors and reproductive behavior in male white rhinoceros (*Ceratotherium simum simum*). *Hormones and Behavior, 45*, 1-9.

Metrione, L. C. (2010), Relationships of Social Behavior and the Captive Environment to Reproduction in Female Southern White Rhinoceros (*Ceratotherium simum simum*), Dissertation, The Ohio State University, Columbus.

Milliken, T., R. H. Emslie, and B. Talukdar (2009), African and Asian rhinoceroses – status, conservation and trade. A report from the IUCN Species Survival Commission (IUNC/SSC) African and Asian Rhino Specialist Groups and TRAFFIC, 1.

Owen-Smith, R. N. (1992), *Megaherbivores: the influence of very large body size on ecology*, reprint, illustrated ed., 388.

Patton, M. L., R. R. Swaisgood, N. M. Czekala, A. M. White, G. A. Fetter, J. P. Montagne, R. G. Rieches, and V. A. Lance (1999), Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior, *Zoo Biol., 18*(2), 111-127.

Radcliffe, R. W., N. M. Czekala, and S. A. Osofsky (1997), combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): Preliminary results, *Zoo Biol., 16*(5), 445-456.

Rookmaaker, K. (2001), The alleged population reduction of the Southern White Rhinoceros (*Ceratotherium simum simum*) and the succesful recovery, *Saugetierkundliche Mitteilungen, 45*(2), 55-70.

Roth, T. L. (2006), A review of the reproductive physiology of rhinoceros species in captivity, *Inter Zoo Yearb, 40*(1), 130-143.

Schwarzenberger, F., R. Francke, and R. Goltenboth (1993), Concentrations of faecal immunoreactive progestagen metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*), *J.Reprod.Fertil., 98*(1), 285-291.

Schwarzenberger, F., C. Walzer, K. Tomasova, J. Vahala, J. Meister, K. L. Goodrowe, J. Zima, G. Strauß, and M. Lynch (1998), Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*), Animal Reproduction Science, 53(1-4), 173-190.

Skinner, J. and R. Smithers (Eds.) (1990), The mammals of the southern African subregion, 234-244, Pretoria, University of Pretoria.

Stoops, M. A., R.D. Pairan, and T.L. Roth (2004), Follicular, endocrine and behavioural dynamics of the Indian rhinoceros (*Rhinoceros unicornis*) estrous cycle. *Reproduction* 128, 843-856.

Swaisgood, R. R., D. M. Dickman, and A. M. White (2006), A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females, *Biological Conservation, 129*(4), 468-476.

Tubbs, C. (2012), Activation of Southern White Rhinoceros (*Ceratotherium simum simum*) Estrogen Receptors by Phytoestrogens: Potential Role in the Reproductive Failure of Captive-Born Females? Endocrinology, 153(3), 1444-1452.

Wittemyer, G., A. Ganswindt, and K. Hodges (2007), The impact of ecological variability on the reproductive endocrinology of wild female African elephants, *Hormones and Behavior, 51*(3), 346-354

Chapter 2

Reproduction in the female white rhinoceros and problems associated with captive breeding: A review

2.1. Background

The family of Rhinocerotidae has hooves with three toes, which makes it a member of the odd-toed ungulate order perissodactyla, together with the Equidae and Tapiridae. Four genera of Rhinocerotidae exist today: *Rhinoceros, Dicerorhinus, Ceratotherium* and *Diceros*, of which only the latter two occur in Africa [Owen-Smith, 1992].

With their large body size, their ability to process a low quality diet and their capability to survive in areas of high habitat disturbance rhinoceroses have persisted over long periods of evolutionary time and are considered as one of the most biologically successful mammals in history [Dinerstein, 2011]. Rhinoceroses also seem to have developed a highly effective immune system, which might have further helped this large mammal survive through time. Known for fighting and using their horns to inflict injuries on each other, they often heal naturally from wounds and infections that would lead to mortality in other ungulates [Estes, 1991; Dinerstein, 2011].

Today, the chances for the rhinoceros to survive have altered, as human interference in the form of illegal poaching for their

Table 2-1. Estimated numbers of white and black rhinos in Africa as of 31 Dec 2012 by country and subspecies [Emslie and Knight, 2014].

Species	White rhino				Black rhino					Total
Subspecies	*C.s.cottoni* (northern)	*C.s.simum* (southern)	TotalWR	Trend	*D.b.bicornis* (south-western)	*D.b.michaeli* (eastern)	*D.b.minor* (southern-central)	TotalBR	Trend	*TotalB&W*
Angola					1			**1**	Min	**1**
Botswana		185	**185**	Up			9	**9**	Up	**194**
Kenya	4	390	**394**	Up		631		**631**	Up	**1,025**
Malawi							26	**26**	Up	**26**
Mozambique		1?	**1**	Down			0?	**0**	?	**1**
Namibia		524	**524**	Up	1,750			**1,750**	Stable	**2,274**
South Africa		18,933	**18,933**	Up	208	68	1,792	**2,068**	Up	**21,001**
Swaziland		84	**84**	Stable			18	**18**	Up	**102**
Tanzania						100	27	**127**	Up?	**127**
Uganda		14	**14**	Up						**14**
Zambia		10	**10**	Up			27	**27**	Stable	**37**
Zimbabwe		284	**284**	Down			424	**424**	Down	**708**
Totals	**4**	**20,424**	**20,429**		**1,959**	**799**	**2,323**	**5,081**		**25,510**

horn is becoming a major threat for all rhinoceros species [Emslie and Knight, 2014]. In Africa, three of the four black rhinoceros subspecies and one of the two white rhinoceros subspecies are currently critically endangered, making them highly conservation dependent. The northern white rhinoceros (*Ceratotherium simum cottoni*), for example, is at the brink of extinction with only 5 individuals remaining [Amin et al., 2006; IUCN, 2014; Emslie and Knight, 2014]. The population of southern white rhinoceros in Africa (*Ceratotherium simum simum*) is currently estimated to be 20,429 of which 93% lives in South Africa (Table 2-1) [Emslie and Knight, 2014].

Managed breeding programs play an important role in the conservation of the white rhinoceros. Many zoological institutions have created breeding programs and work together with the goal to create a protected population of white rhinoceros, which can potentially be used to reintroduce individuals into the wild. Today, numbers of white rhinoceros in captivity are estimated to be 700 [Bertschinger, 1994]. This

population is however not self-sustaining and new individuals are continuously imported from the wild [Foose and Wiese, 2006]. A major threat influencing this negative growth rate is the failure to breed successfully amongst white rhinoceroses in captivity [Swaisgood et al., 2006], an observation uncommon in free-living populations [Bertschinger, 1994].

2.2. Anatomy of the reproductive system

Reproductive tract morphology in the white rhinoceros is similar to the black rhinoceros. Specific anatomic measurements of the reproductive tract are provided in Table 2-2 [Godfrey et al., 1991]. The uterus of the white rhinoceros is bicornuate with a short body and relatively long horns, thus closely resembling the uterus of the canidae and the suidae (Fig. 2-1 and 2-2) and the cervix is narrow due to the presence of eccentric rings [Schaffer, 2001; Godfrey et al., 1991]. This, in combination with a smooth, long vagina with large longitudinal folds, has implications for potential techniques used to assist breeding, such as rectal palpation, ultrasonography, embryo transfers and artificial insemination [Schaffer, 2001; Godfrey et al., 1991]. The ovaries are located cranial of the first rib and caudal of the kidneys [Miller, 2014]. The white rhinoceros seems to lack an ovulation fossa, a specific area found on the ovary of the mare where preovulatory follicles migrate to before ovulation and it seems that the rhinoceros ovulates at several sites on the surface[Sorensen, 1979; Godfrey et al., 1991].

Table 2-2. Measurements of reproductive tracts from black and white rhinoceros [Godfrey et al., 1991]

	Black rhinoceros			White rhinoceros	
Studbook No.	2058	367	38	751	45
Age	7 days	3 years	28 years	21 years	27 years
Length of					
Vestibule[a]					
cm	6.8	—[d]	15.5	19.6	14.3
%[b]	19.6	—	14.5	18.5	15.3
Vagina[c]					
cm	14.1	25.0	22.1	30.1	19.0
%	40.6	34.5	20.7	28.4	20.3
Cervix					
cm	2.8	10.5	17.0	13.0	12.0
%	8.1	14.5	15.9	12.3	12.9
Uterine body					
cm	.5	2.7	5.5	3.5	7.5
%	1.4	3.7	5.2	3.3	8.0
Uterine horns[e]					
cm	10.5	34.3	46.5	39.9	40.6
%	30.3	47.3	43.6	37.6	43.5
Total (cm)	34.7	72.5	106.6	106.1	93.4
Diameter of uterine horn[e] (cm)	0.5	1.5	2.0	3.1	4.0

[a]Vulva to distal surface hymen.
[b]Percent of total tract length.
[c]Proximal surface of hymen to external cervical os.
[d]Vulva was missing from tract, so total length is reported without vestibule.
[e]Represents average of left and right horns.

A hymen just cranial of the urethral opening may be present in nulliparous females, which does not rupture until the female gives birth [Schaffer, 2001; Godfrey et al., 1991; Miller, 2014]. This membrane stays intact during sexual intercourse, unlike the situation in most other mammals. However, a similar structure has been reported in the Asian and African elephant and is used in these species to predict whether a female is primigravid [Balke et al., 1988; Schaffer, 2001; Godfrey et al., 1991].

Like in most hooved mammals, the adult female white rhinoceros has two inguinal mammae [Schaffer, 2001].

Figure 2-1. Dorsal orientation of the reproductive tract of the female white rhinoceros. O = Ovary; U = Uterine horn, C = cervix; V = vagina; L = vulva [Schaffer et al., 2001]

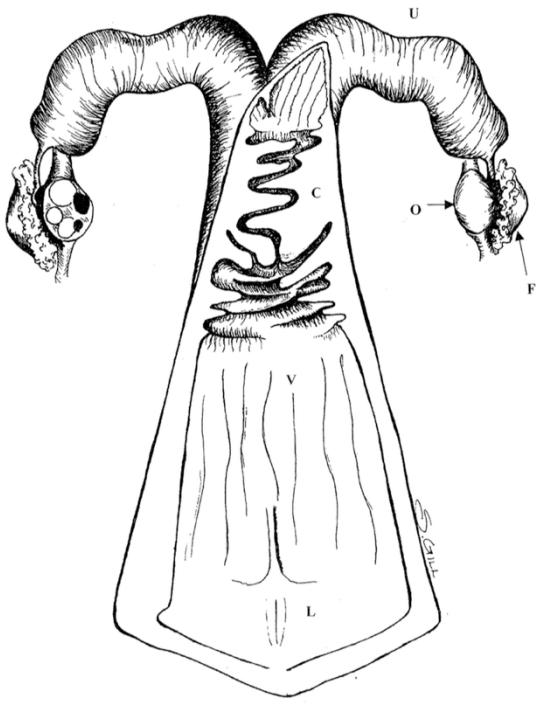
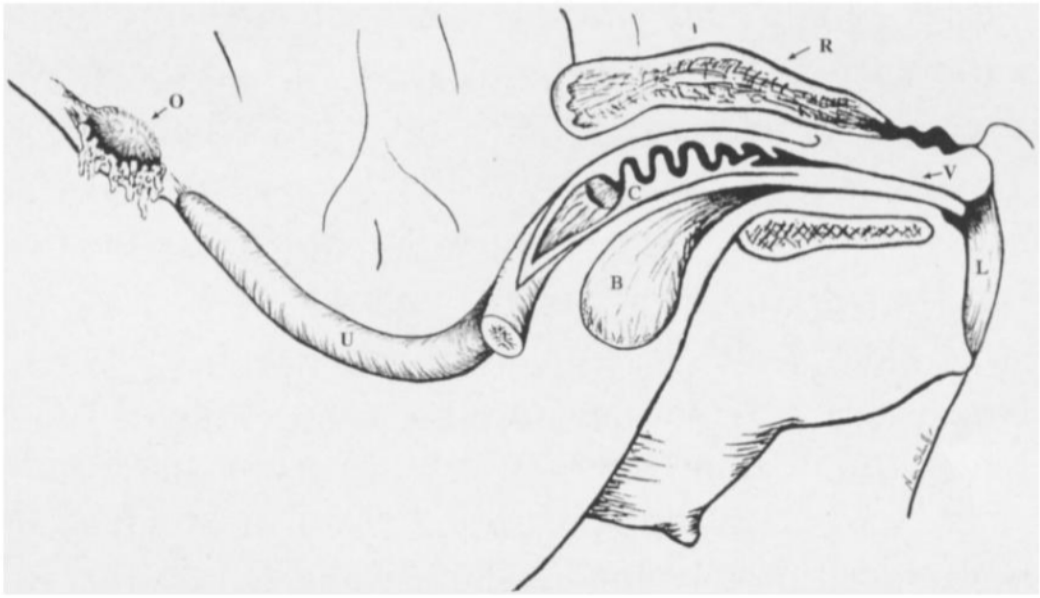


Figure 2-2. Abdominal orientation of the reproductive tract of the female white rhinoceros. O = Ovary; U = Uterine horn, C = cervix; V = vagina; L = vulva; B = Urinary bladder; R = Rectum [Schaffer et al., 2001]



2.3. Basic data on the reproductive physiology

2.3.1. Reproductive behavior

Unlike the black rhinoceros with its solitary habits [Goddard, 1967; Schenkel et al., 1969], the white rhinoceros' social organization appears to be unique [Owen-Smith, 1975; Leuthold, 1977] and its complexity has not yet been entirely unraveled. Previous studies reported stable groups (> 1 month) of up to six individuals [Owen-Smith, 1975], consisting of territorial bulls, sub-adults, cows and their offspring [Skinner and Smithers, 1990]. Rhinoceros urine and feces play an important role in communication. Territorial bulls mark their territories by spray urination while scraping with their hind legs and by creating well-defined middens where communal defecation takes place [Skinner and Smithers, 1990]. When encountering a scent of urine or feces from another individual, both males and females normally sniff the area to assess reproductive status [Miller, 2014]. When females are in estrus they are closely attended by the dominant bull, which often goes accompanied with fighting and can even lead to serious external and sometimes internal injuries [Skinner and Smithers, 1990; Dixson et al., 2003]. The dominant bull will stay within close proximity of the female and repeatedly approach her for a period of up to 20 hours before mating occurs, which has a duration of approximately 20-30 minutes [Dixson et al., 2003]. Sub-adult bulls do not approach females in estrus and will normally be accepted in the territory of a dominant bull [Dixson et al., 2003].

2.3.2. Reproductive cycle

Our knowledge of the reproductive endocrinology of the white rhinoceros is still fragmentary. Measurement of fecal and urinary progestagen metabolite (fPM) concentrations, feasible as a result of the validation of group-specific enzyme immune assays for measuring fecal immunoreactive pregnanes, has recently made it possible to characterize the estrous cycle and assess luteal activity in animals that do not lend themselves well for regular blood sampling, such as the rhinoceros [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001]. However, many contradictions exist about the cycle length of the white rhinoceros. In the black rhinoceros, the estrous cycle has been reported to be approximately 26 days [Schwarzenberger et al., 1993; Brown et al., 2001; Garnier et al., 2002]. In the white rhinoceros, data reported in previous studies seems more cryptic. One observational study recorded estrous cycles of approximately 30 days in 3 free-living white rhinoceros females [Owen-Smith, 1992]. More recently, several sound scientific studies monitoring hormone metabolites in feces and urine have focused on characterizing the estrous cycle of the white rhinoceros in captivity, and the data collectively suggests that there are two types of cycle, differing with regard to luteal phase length: a “short” cycle of 30-35 days and a “long” cycle of 65-70 days [Hindle et al., 1992; Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001]. It is believed that only the shorter cycle results in pregnancy, as pregnancy after mating associated with a 65–70 day cycle has not yet been documented, suggesting that long cycles have a

pathological origin [Schwarzenberger et al., 1998; Brown et al., 2001; T. L. Roth, 2006]. On the other hand, it is not known whether wild rhinoceros females show similar characteristics, which means we do not have a solid foundation of the normal reproductive biology of the species [Swaisgood et al, 2006]. The collection of regular blood samples for progesterone measurements would require sedation and capture of the individuals on a weekly basis, a highly impractical proposition. While the collection of regular fecal samples and analyses of progestagen metabolite profiles would offer a more realistic alternative for assessment of luteal activity in females living in the wild, such data have not been published to date [Swaisgood et al., 2006].

Another observation in captivity is that approximately 50% of the adult white rhinoceros females within all age classes show long periods of acyclicity [Brown et al., 2001; Hermes et al., 2006]. Moreover, while most young anestrous females show ovarian activity with regular follicle waves, ovulation often remains absent in these individuals [Radcliffe et al., 1997]

2.3.3. Pregnancy and lactation

Based on studies in captivity, gestation in the white rhinoceros is estimated to be 16-18 months [Fouraker and Wagener, 1996; Patton et al., 1999]. Data from captive females during pregnancy shows that fPM concentrations start elevating above luteal values around two to five month post ovulation and this non-invasive technique can therefore be used as a method to detect pregnancy [Schwarzenberger et al., 1998; Hermes et al., 2012].

The white rhinoceros is a non-seasonal breeder and calves can be born at any time of the year [Patton et al., 1999; Brown et al., 2001]. However, some studies have suggested that the onset of estrus might be stimulated by new flushes of green grass after rainy season, resulting in a mating peak in spring in some areas [Owen-Smith, 1992]. Close to parturition, females in the wild normally separate themselves from other rhinoceroses and hide away in thick vegetation. During this time they also chase away their previous calf [Skinner and Smithers, 1990; Owen-Smith, 1992]. Approximately 30 days prior to calving, swelling of the mammae and a mild vagina prolapse can be visible and calving normally occurs rapidly within 1-3 hours [Skinner and Smithers, 1990]. In captivity, fPM concentrations drop to baseline values rapidly after parturition and remain low for the first 3–5 months after parturition [Brown et al., 2001]. Additionally, Schwarzenberger et al. (1998) found that females display a post-partum estrus in the absence of mating approximately 1 month after birth, similar to the “foalestrus” in the mare [Schwarzenberger et al., 1993; McCue et al., 2007]. In the wild, behavioral studies have reported that females start cycling from 6-12 months post partum [Owen-Smith, 1975]. A newborn calf weighs around 40 kg [Bertschinger, 1994]. One study reported that calves shed their first skin from 1.5 to 4 months of age, resulting in a new skin that is whiter in color. A second less obvious shedding period was also observed in this study, suggesting that the shedding of skin takes place more than once [Bigalke et al., 1950]. The average lactation period in the wild white rhinoceros is 19 months for female calves and 27 months for male calves [White et al., 2007], although calves accompany

their mothers until they are about two to three years of age [Skinner and Smithers, 1990]. Calves will start nibbling grass from an age of 2 months and will not drink water from natural resources until they are approximately 5 months old [Owen-Smith, 1975]. Inter-calving periods in the wild normally range from 2.63 – 3.45 years [Owen-Smith, 1992; Bertschinger, 1994], although shorter periods have been documented [Owen-Smith, 1992].

2.3.4. Juvenile development

In the wild, young females have their first estrus around 4-6 years of age [Condy, 1973; Owen-Smith, 1975]. Sub-adult bulls start to become solitary between 8-10 years but do not display territorial behavior until the age of 12.5 years [Condy, 1973; Owen-Smith, 1975; Owen-Smith, 1992].

2.4. Breeding soundness in captivity

2.4.1. Pregnancy failure

Ex situ breeding programs are valuable tools to serve as genetic reservoirs while providing animals for reintroduction [Emslie and Brooks, 1999; Hildebrandt et al., 2003]. Retrospective studies evaluating reproductive success in the captive white rhinoceros population, differentiating between females that were imported from the wild (F0) and captive-born females (F1, F2, etc.), have revealed that a large group of white rhinoceros females fails to reproduce [Swaisgood et al., 2006; Reid et al., 2012]. Data shows that less then 50% of the females ever reproduced, and this number is even lower in the F1 generation,

of which only 39% ever produced a calf [Swaisgood et al., 2006]. The consequence is a negative growth rate of -3.5% of the entire population, resulting in a population that is unable to sustain itself without new imports from the wild [Foose and Wiese, 2006].

In recent years, a lot of attention has been given to the potential underlying mechanisms as well as causative factors responsible for this reproductive impairment, but findings are still unclear and controversial [Schwarzenberger et al., 1999; Carlstead and Brown, 2005; Swaisgood et al., 2006; Metrione and Harder, 2011; Tubbs, 2012]. Studies reported that male fertility in captivity is generally good and that copulation happens normally [Hermes et al., 2005; Swaisgood et al., 2006]. This indicates that post-copulatory mechanisms in the female are most likely responsible for observed pregnancy failure in the captive population of the white rhinoceros.

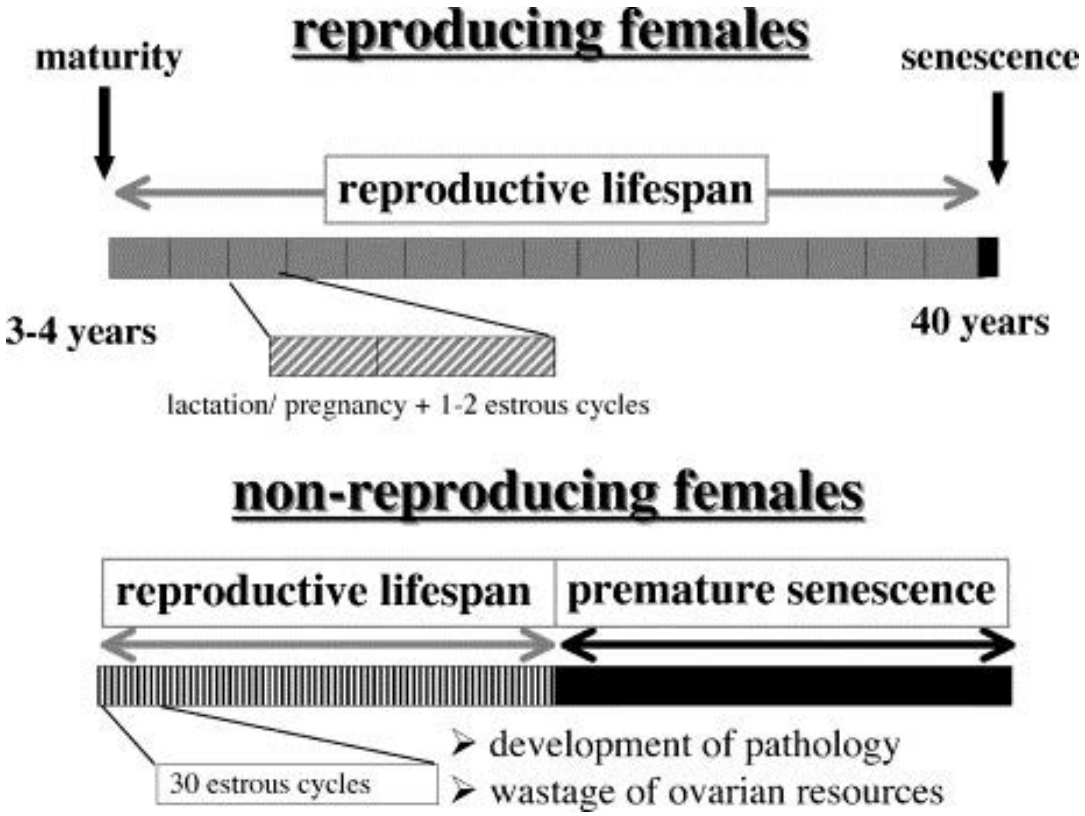
2.4.2. Reproductive pathologies

In addition to the observed pregnancy failure, it has been documented that many adult white rhinoceros females in captivity show reproductive tract pathologies, which might further negatively influence breeding output [Schaffer, 2001; Godfrey et al., 1991; Hermes et al., 2004; Hermes et al., 2006]. Hermes et al. 2006 reported a large number of various seemingly abnormal observations in the reproductive system of adult female white rhinoceroses, which included endometrial hyperplasia, ovarian cysts (2-15 cm) and tumors in the uterus, ovaries, cervix and vagina. This study also found that these findings are hormone-dependent and associated with age and suggested they are likely

the result rather than the cause of long non-reproductive periods (Fig. 2-3) [Hermes et al., 2004; Hermes et al., 2006]. Although infectious diseases can cause similar problems, there are no indications to assume underlying infectious causes in these white rhinoceros females [Hermes et al., 2006]. The association between ovulation frequency and genital pathologies and ovarian cancer in cycling yet non-reproducing females has been a well-established phenomenon amongst domesticated and captive-born species [Fathalla, 1971; Okkens et al., 1997; Maruo et al., 2000; Walker, 2002; Agnew et al., 2004]. Similarly, some studies have reported a correlation between the amount of non-conceptive cycles and the onset of menopause in humans [Sowers and La Pietra, 1995; Cramer et al., 1995]. Years of reproductive cycling without interruption by anovulatory periods during pregnancy and lactation may result in oocyte depletion and loss of ovarian follicular function [Fathalla, 1971]. The postovulatory repetitive wounding and cell proliferation that occurs post-ovulation is suggested to lead to mutations that can cause tumors [Godwin et al., 1992]. In addition, a continuous production of estrogens can lead to progressive cystic endometrial hyperplasia and/or endometrial adenoma, which were both documented as commonly present in aged white rhinoceros females in captivity [Hermes et al., 2006]. Abnormal growth of the endometrium may subsequently prevent conception and embryo implantation in uteri [Godfrey et al., 1991]. It is recommended to breed female white rhinoceroses before 10 years of age, which is thought to prevent the development of ovarian and uterine pathologies [Versteege, 2012].

In order to further understand underlying mechanisms responsible for the genital pathologies observed in captive females, it would be useful to confirm estrogen overexposure by monitoring estrogen production non-invasively. However, to date there are no hormone assays available that can reliably measure estrogens in the feces of the white rhinoceros [Schwarzenberger et al., 1998; Brown et al., 2001; T. L. Roth, 2006].

Figure 2-3. Schematic graph of the reproductive aging process in reproducing and non-reproducing female rhinoceroses in captivity [Hermes et al., 2004]. In successfully reproducing females, the reproductive lifespan consists of a more or less fixed series of ovulations followed by pregnancy and lactation, and is spread out between age of maturity (3-4 years) and age of senescence (± 40 years). In contrast, non-reproducing females suffering from pregnancy failure ovulate much more frequently and reach senescence at a much earlier stage of life (premature senescence), resulting in a shorter reproductive lifespan and an increased risk for reproductive pathologies.



2.4.3. Potential factors affecting reproductive success

Ex situ breeding of the white rhinoceros comes with three major unexplained observations in the majority of females: 1) Pregnancy failure; 2) Aberrant cycling and acyclicity; and 3) Reproductive pathologies. Of these three, the latter seems to be a result of the first two observations [Hermes et al., 2006]. Two underlying post-copulatory mechanisms have been proposed to explain the "long cycles" identified in captive females: 1) early embryonic death [Radcliffe et al., 1997], and 2) luteinized follicular tissue [Adams et al., 1991; Radcliffe et al., 1997; Schwarzenberger et al., 1998]. Additional investigations are needed however to test these hypotheses. Numerous studies in recent years have investigated effects of environmental factors on white rhinoceros breeding success in captivity, but no clear correlation has been discovered. One study investigated the possible role of stress by examining fecal corticoid profiles in relation to cyclicity, behavior and environmental factors and found higher corticoid variability, more stereotypic pacing and less olfactory behavior in non-cycling females compared to cycling females, suggesting that acyclicity might be associated with stressors in the environment [Carlstead and Brown, 2005]. In 2011, Metrione et al. published data from a dissertation describing average corticosterone concentrations for females housed in a variety of environmental conditions, and assessing the effects of factors such as group size and the number of males available [Metrione and Harder, 2011]. No significant relationships were found in this study. In another approach to the breeding problem, Tubbs (2012) considered the diet of the majority of females, hypothesizing

that a high amount of phytoestrogens present in the diet of white rhinoceroses might play a role in reproductive failure [Tubbs, 2012]. Phytoestrogens are natural compounds found in certain legumes such as alfalfa and soy and can mimic endogenous estrogens in the body by binding and activating estrogen receptors in the white rhinoceros, potentially resulting in estrogen overexposure [Tubbs, 2012]. Both alfalfa and soy products are commonly present in high quantities in the diet of captive white rhinoceroses [Tubbs, 2012], and additional investigations into the correlation between dietary exposure and breeding success are required to further test this hypothesis. Several other environmental factors may be responsible for reproductive failure, including enclosure size, animal density and social group structures. As the white rhinoceros is a species with a highly developed and complex social system, more so than all the other rhinoceros species [Owen-Smith, 1975], it is not unlikely that a social factor with behavioral implications is responsible for the problem, which is being supported by documented cases of females returning to regular cycling after being transported to other facilities or introduced to a new male [Patton et al., 1999; Hermes et al., 2006]. In order to establish a self-sustaining *ex situ* breeding population of white rhinoceros that can assist in the conservation of this species, further research is needed into the underlying mechanisms and causes for poor reproductive health in the white rhinoceros.

2.4.4. Reproduction in the wild

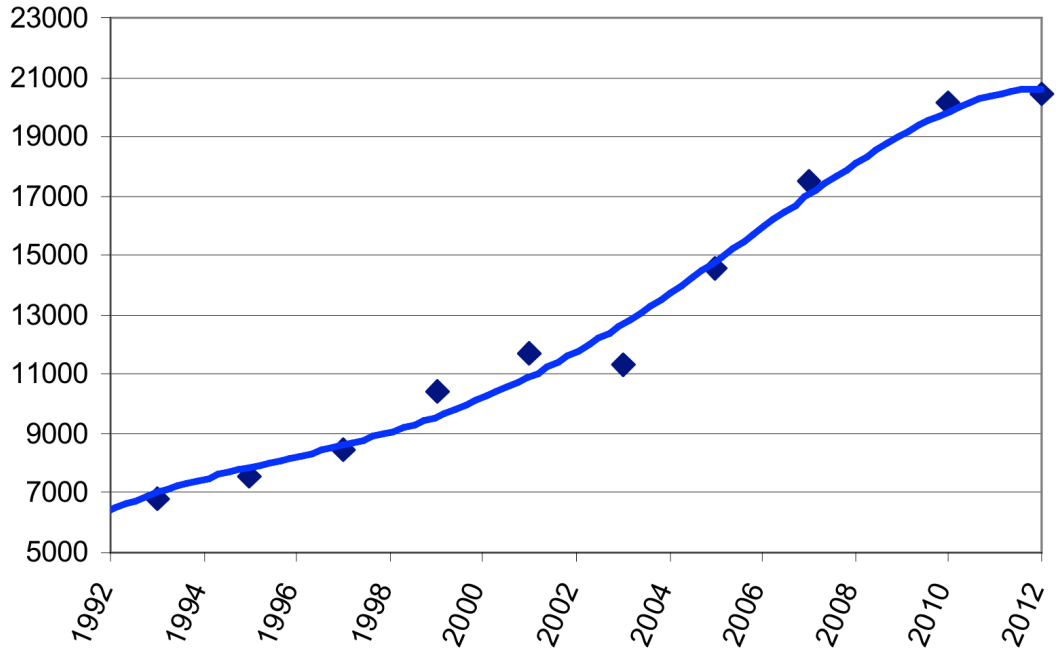
Despite the daily loss of white rhinoceroses in Africa to

poaching, the wild population of white rhinoceroses is currently still growing [Emslie and Knight, 2014]. Recent continental rhinoceros population estimates however show that this growth is slowing in response to the rise of poaching incidents (Fig. 2-4) [Emslie and Knight, 2014]. Failure to reproduce and obstetrical problems in wild females are not common [Owen-Smith, 1975; Silberman and Fulton, 1979; Kretzschmar et al., 2004]. It would therefore be very useful to compare ovarian cycle characteristics as well as female genital health of wild individuals with problem females in captivity.
Unfortunately, previously published reproductive endocrinology and genital health studies in the white rhinoceros virtually all presented data from captive individuals, mainly due to the **remoteness** and elusiveness of rhinoceroses living in the wild. It is therefore not known with certainty whether females in the wild exhibit a natural variation in cycle length and/or experience periods of acyclicity and whether aged females in the wild are sensitive to the development of reproductive tract abnormalities. A study focusing on the relationship between environmental factors and reproductive behaviour in free-living male white rhinoceroses suggested a correlation between the number of offspring per male and vegetation structure [Kretzschmar, 2004]. However, more research is needed on the relationship between the natural environment and reproduction in free-living females if we are to gain a better understanding and thus be able to interpret findings in captivity.

2.5. Advanced reproductive technologies

Much progress has been made in the last two decades towards the use of ultrasound techniques in different rhinoceros species in captivity to characterize reproductive events [Adams et al., 1991; Radcliffe et al., 1997; T. Roth et al., 2001; T. L. Roth et al., 2004]. Specific challenges such as the hymen and the long cervix were overcome with the development of specialized angled ultrasound probe extensions of various sizes for special use in the white rhinoceros [Godfrey et al., 1991; Hermes et al., 2006; Hermes et al., 2007].

Figure 2-4. White rhinoceros population trends 1991-2012. Changes in estimated numbers of white rhinoceroses in Africa since 1991 with fitted second-order polynomial trend line (IUCN SSC AfRSG data) [Emslie and Knight, 2014].



Ultrasound monitoring during pregnancy now enables precise knowledge of the timing of ovulation as well as an accurate prediction of parturition, enabling appropriate preparation for

intervention and creating opportunities in the development of assisted reproduction technology (ART) such as artificial insemination (AI) [Hermes et al., 2007].

As a tool that can considerably optimize reproductive performance, the use of AI in captive rhinoceroses is highly desired, particularly in the white rhinoceros [Pukazhenthi et al., 2005; Hermes et al., 2007; Wildt et al., 2010; Schwarzenberger and Brown, 2013]. Three successful pregnancies have so far been reported following AI with fresh semen after a natural ovulation in the white rhinoceros [Hildebrandt et al., 2007; Hermes et al., 2007]. However, in order to successfully inseminate females at the right time on a regular basis it is important that the moment of ovulation can be predicted accurately [Hildebrandt et al., 2007], which seems to be challenging in the white rhinoceros, especially since the majority of females are acyclic or do not cycle regularly [Radcliffe et al., 1997; Hermes et al., 2006]. A reliable protocol to induce estrus and ovulation in the white rhinoceros is therefore desirable. Several cases have been published using different combinations of treatment agents [Godfrey et al., 1990; Walzer and Schwarzenberger, 1995; Schwarzenberger et al., 1998; Hermes et al., 2006; Hildebrandt et al., 2007]. Most of these protocols were, however, relatively unsuccessful with a maximum ovulation rate of only 30% [Hermes et al., 2006]. Hermes et al. (2012) presented a retrospective study evaluating induction of ovulation in the white rhinoceros using the synthetic progestin chlormadinone acetate (CMA) in combination with hCG or the GnRH analogue deslorelin [Hermes et al., 2012]. And although cyclic activity was initiated successfully in this

study, estrus induction failed to induce a lasting effect [Hermes et al., 2012] and additional investigations are required to further develop a feasible and reliable treatment protocol for estrus and ovulation induction in the white rhinoceros. More advanced technologies for improving breeding success include embryo transfer and *in vitro* technologies such as *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI). Such tools are especially useful for the preservation of genetic material of non-reproducing white rhinoceros females that otherwise would not be conserved. This is of particular importance for the critically endangered northern subspecies, as the last few females remaining of this subspecies are infertile as a result of reproductive aging [Hermes et al., 2007]. Most attempts to superstimulate white rhinoceroses and collect oocytes have failed due to technical problems related to challenging anatomical features [Hermes et al., 2007]. However, recent transrectal ultrasound guided trials in two black rhinoceroses and one white rhinoceros proved to be promising, as this trial resulted in the first successful oocyte recoveries from live donors [Hermes et al., 2009]. Recovered oocytes were subsequently used in IVM trials, resulting in the first black rhinoceros IVF embryo ever produced [Hermes et al., 2009]. So far, no attempts to transfer embryos in the white rhinoceros have been reported [Hermes et al., 2007; Hermes et al., 2009].

2.6. Conclusion

This review functioned as a general overview of known information related to reproduction in the white rhinoceros. An

understanding of the reproductive anatomy and physiology in the white rhinoceros as well as the poorly understood reproductive soundness seen in captivity is essential when pursuing strategies to maximize breeding output, which makes this topic a prime domain for current white rhinoceros research.

With further research, integrating progressive advances made in the development of assisted reproduction into the management regime for the white rhinoceros, great potentials arise for improving breeding success and reducing the occurrence of reproductive pathologies in this species. Before such tools can be used on a regular basis it remains of critical importance that we increase our knowledge on the reproductive physiology in the white rhinoceros and succeed in detecting the causative factors in the captive environment that are responsible for the low reproductive rates seen in captivity. By increasing our understanding of the complex physiological mechanisms that drive reproduction in this species, we will be better able to facilitate reproductive success and even survival of the white rhinoceros.

2.7. References

Adams, G. P., E. D. Plotka, C. S. Asa, and O. J. Ginther (1991), Feasibility of characterizing reproductive events in large nondomestic species by transrectal ultrasonic imaging, Zoo Biol., 10(3), 247-259.

Agnew, D. W., L. Munson, and E. C. Ramsay (2004), Cystic Endometrial Hyperplasia in Elephants, Veterinary Pathology Online, 41(2), 179-183.

Amin, R., K. Thomas, R. H. Emslie, T. J. Foose, and N. V. Strien (2006), An overview of the conservation status of and threats to rhinoceros species in the wild, International Zoo Yearbook, 40(1), 96-117.

Balke, J. M. E., W. J. Boever, M. R. Ellersieck, U. S. Seal, and D. A. Smith (1988), Anatomy of the reproductive tract of the female African elephant (*Loxodonta africana*) with reference to development of techniques for artificial breeding, Journal of Reproduction and Fertility, 84(2), 485-492.

Bertschinger, H. J. (1994), Reproduction in black and white rhinos: a review, Proceedings of a symposium on rhinos as game ranch animals. Onderstepoort, Republic of South Africa, 9-10 September 1994, 96-99.

Bigalke, R., T. Steyn, D. D. Vos, and K. D. Waard (1950), Observations on a juvenile female square-lipped or white rhinoceros (*Ceratotherium simum simum* (Burch.) in the National Zoological Gardens of South Africa. Proceedings of the Zoological Society of London, 120(3), 519-528.

Brown, J. L., A. C. Bellem, M. Fouraker, D. E. Wildt, and T. L. Roth (2001), Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring, Zoo Biol., 20(6), 463-486.

Carlstead, K. and J. L. Brown (2005), Relationships between patterns of Fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros, Zoo Biol., 24(3), 215-232.

Condy, P. R. (1973), The population status, social behaviour, and daily activity pattern of the white rhino (*Ceratotherium simum simum*) in Kyle National Park, Rhodesia. University of Rhodesia, Division of Biological Sciences, Salisbury.

Cramer, D. W., H. Xu, and B. L. Harlow (1995), Does “incessant” ovulation increase risk for early menopause? Obstet. Gynecol., 172(2, Part 1), 568-573.

Dinerstein, E. (2011), Family Rhinocerotidae (rhinoceroses). In Handbook of the Mammals of the World, vol. 2, edited by D. Wilson and R. Mittermeier, 182-204, Barcelona, Lynx Editions.

Dixson, A., N. Harvey, M. Patton, and J. Setchell (2003), **Behaviour and reproduction**. In 'Reproductive Science and Integrated Conservation'. (Eds W. V. Holt, A. R. Pickard, X. C. Rodger and D. E. Wildt), 24-41. Cambridge University Press.

Emslie, R. H. and M. Brooks (1999), African Rhino: Status Survey and Conservation Action Plan, IUCN/SSC African Rhino Specialist Group, Gland, Switzerland and Cambridge, UK.

Emslie, R. H. and M. H. Knight (2014), Update on African rhino status and trends: from IUCN SSC African Rhino Specialist Group (AfRSG), in Report to CITES Standing Committee 65th meeting, 1-6.

Estes, R. D. (1991), The Behavior Guide to African Mammals: Including Hoofed Mammals, Carnivores, Primates, University of California Press, Berkeley.

Fathalla, M. (1971), Incessant ovulation—a factor in ovarian neoplasia? The Lancet, 298(7716), 163.

Foose, T. J. and R. J. Wiese (2006), Population management of rhinoceros in captivity, International Zoo Yearbook, 40(1), 174-196.

Fouraker, M. and T. Wagener (1996), AZA Rhinoceros husbandry resource manual, 1-67.

Garnier, J., W. Holt, and P. Watson (2002), Non-invasive assessment of oestrous cycles and evaluation of reproductive seasonality in the female wild black rhinoceros (*Diceros bicornis minor*), Reproduction, 123(6), 877-889.

Goddard, J. (1967), Home range, behaviour, and recruitment rates of two black rhinoceros populations, Afr. J. Ecol., 5(1), 133-150.

Godfrey, R. W., C. E. Pope, B. L. Dresser, B. D. Bavister, J. C. Andrews, and J. H. Olsen (1990), An attempt to superovulate a southern white rhinoceros (*Ceratotherium simum simum*), Theriogenology, 33, 231.

Godfrey, R. W., C. E. Pope, B. L. Dresser, and J. H. Olsen (1991), Gross anatomy of the reproductive tract of female black (*Diceros bicornis michaeli*) and white rhinoceros (*Ceratotherium simum simum*), Zoo Biol., 10(2), 165-175.

Godwin, A. K., J. R. Testa, L. M. Handel, Z. Liu, L. A. Vanderveer, P. A. Tracey, and T. C. Hamilton (1992), Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer, J. Natl. Cancer Inst., 84(8), 592-601.

Hermes, R., F. Göritz, T. J. Portas, B. R. Bryant, J. M. Kelly, L. J. Maclellan, T. Keeley, F. Schwarzenberger, C. Walzer, A. Schnorrenberg, R. E. Spindler, J. Saragusty, S. Kaandorp, and T. B. Hildebrandt (2009), Ovarian superstimulation, transrectal ultrasound-guided oocyte recovery, and IVF in rhinoceros, Theriogenology, 72(7), 959-968.

Hermes, R., F. Göritz, W. Streich, and T. Hildebrandt (2007), Assisted Reproduction in Female Rhinoceros and Elephants – Current Status and Future Perspective, Reproduction in Domestic Animals, 42(s2), 33-44.

Hermes, R., T. B. Hildebrandt, and F. Göritz (2004), Reproductive problems directly attributable to long-term captivity–asymmetric reproductive aging, Anim. Reprod. Sci., 82-83, 49-60.

Hermes, R., T. B. Hildebrandt, C. Walzer, F. Göritz, C. Gray, C. Niemuller, and F. Schwarzenberger (2012), Estrus induction in white rhinoceros (*Ceratotherium simum*), Theriogenology, 78(6), 1217-1223.

Hermes, R., T. B. Hildebrandt, S. Blottner, C. Walzer, S. Silinski, M. L. Patton, G. Wibbelt, F. Schwarzenberger, and F.

Göritz (2005), Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation, Theriogenology, 63(1), 219-238.

Hermes, R., T. B. Hildebrandt, C. Walzer, F. Göritz, M. L. Patton, S. Silinski, M. J. Anderson, C. E. Reid, G. Wibbelt, K. Tomasova, and F. Schwarzenberger (2006), The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*), Theriogenology, 65(8), 1492-1515.

Hildebrandt, T. B., J. L. Brown, R. Hermes, and F. Göritz (2003). Ultrasound for analysis of reproductive function in wildlife species. In 'Reproductive Science and Integrated Conservation'. (Eds W. V. Holt, A. R. Pickard, X. C. Rodger and D. E. Wildt), 166-182. Cambridge University Press.

Hildebrandt, T. B., R. Hermes, C. Walzer, E. Sós, V. Molnar, L. Mezösi, A. Schnorrenberg, S. Silinski, J. Streich, F. Schwarzenberger, and F. Göritz (2007), Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation, Theriogenology, 67(9), 1473-1484.

Hindle, J. E., E. Most, and J. K. Hodges (1992), Measurement of urinary oestrogens and 20{alpha}-dihydroprogesterone during ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses, J. Reprod. Fertil., 94(1), 237-249.

IUCN, 2014: *IUCN Red List of Threatened Species*. [Available online at www.iucnredlist.org].

Kretzschmar, P., U. Gansloßer, and M. Dehnhard (2004), Relationship between androgens, environmental factors and reproductive behavior in male white rhinoceros (*Ceratotherium simum simum*), Hormones and Behavior, 45(1), 1-9.

Leuthold, W. (1977), African ungulates, Zoophysiology and ecology, 8, 1-307.

Maruo, T., H. Matsuo, T. Samoto, Y. Shimomura, O. Kurachi, Z. Gao, Y. Wang, I. M. Spitz, and E. Johansson (2000), Effects of progesterone on uterine leiomyoma growth and apoptosis, Steroids, 65(10–11), 585-592.

McCue, P. M., J. Samper, J. Pycock, and A. McKinnon (2007), Ovulation failure, Current therapy in equine reproduction, 83-85.

Metrione, L. C. and J. D. Harder (2011), Fecal corticosterone concentrations and reproductive success in captive female southern white rhinoceros, Gen. Comp. Endocrinol., In Press, Uncorrected Proof.

Miller, R. E. (2014), Rhinoceridae (Rhinoceroses), in Zoo and Wild Animal Medicine, vol. 8, edited by M. E. Fowler and R. E. Miller, 545-546, Philadelphia, Saunders.

Okkens, A. C., H. S. Kooistra, and R. F. Nickel (1997), Comparison of long-term effects of ovariectomy versus ovariohysterectomy in bitches, J. Reprod. Fertil. Suppl., 51, 227-231.

Owen-Smith, R. N. (1992), Megaherbivores: the influence of very large body size on ecology, Cambridge University Press.

Owen-Smith, R. N. (1975), The Social Ethology of the White Rhinoceros *Ceratotherium simum* (Burchell 1817*), Zeitschrift für Tierpsychologie, 38(4), 337-384.

Patton, M. L., R. R. Swaisgood, N. M. Czekala, A. M. White, G. A. Fetter, J. P. Montagne, R. G. Rieches, and V. A. Lance (1999), Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior, Zoo Biol., 18(2), 111-127.

Pukazhenthi, B., P. Comizzoli, A. J. Travis, and D. E. Wildt (2005), Applications of emerging technologies to the study and

conservation of threatened and endangered species, Reproduction, Fertility and Development, 18(2), 77-90.

Radcliffe, R. W., N. M. Czekala, and S. A. Osofsky (1997), Combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): Preliminary results, Zoo Biol., 16(5), 445-456.

Reid, C. E., L. Versteege, A. Ludwig, and R. Hermes (2012), Analysis of the European captive Southern white rhinoceros *Ceratotherium simum simum* population using the International Studbook for the African White Rhinoceros (2001-2004) and integrating reproductive health findings, International Zoo Yearbook.

Roth, T. L. (2006), A review of the reproductive physiology of rhinoceros species in captivity, International Zoo Yearbook, 40(1), 130-143.

Roth, T. L., H. L. Bateman, J. L. Kroll, B. G. Steinetz, and P. R. Reinhart (2004), Endocrine and ultrasonographic characterization of a successful pregnancy in a Sumatran rhinoceros (*Dicerorhinus sumatrensis*) supplemented with a synthetic progestin, Zoo Biol., 23(3).

Roth, T., J. O'Brien, M. McRae, A. Bellem, S. Romo, J. Kroll, and J. Brown (2001), Ultrasound and endocrine evaluation of the ovarian cycle and early pregnancy in the Sumatran rhinoceros, *Dicerorhinus sumatrensis*, Reproduction, 121(1), 139-149.

Schaffer, N. E., G. L. Foley, S. Gill, and C. E. Pope (2001), Clinical Implications of Rhinoceros Reproductive Tract Anatomy and Histology, Journal of Zoo and Wildlife Medicine, 32(1), 31-46.

Schenkel, R., L. Schenkel-Hulliger, and L. S. Hulliger (1969), Ecology and behaviour of the black rhinoceros (*Diceros bicornis L.*): a field study, vol. 5, Paul Parey.

Schwarzenberger, F. and J. Brown (2013), Hormone monitoring: an important tool for the breeding management of wildlife species, Wiener Tierärztliche Monatsschrift, 100, 209-225.

Schwarzenberger, F., R. Francke, and R. Goltenboth (1993), Concentrations of faecal immunoreactive progestagen metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*), J. Reprod. Fertil., 98(1), 285-291.

Schwarzenberger, F., C. Walzer, K. Tomasova, J. Vahala, J. Meister, K. L. Goodrowe, J. Zima, G. Strauß, and M. Lynch (1998), Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*), Animal Reproduction Science, 53(1-4), 173-190.

Schwarzenberger, F., C. Walzer, K. Tomasova, J. Zima, F. Göritz, R. Hermes, and T. B. Hildebrandt (1999), Can the problems associated with the low reproductive rate in captive white rhinoceroses *(Ceratotherium Simum)* be solved within the next 5 years? Erkrankungen der Zootiere, 39, 283-289.

Silberman, M. and R. Fulton (1979), Medical problems of captive and wild rhinoceros: A review of the literature and personal experiences, The Journal of Zoo Animal Medicine, 6-16.

Skinner, J. and R. Smithers (Eds.) (1990), The mammals of the southern African subregion, 234-244, Pretoria, University of Pretoria.

Sorensen, A. M.,jr. (1979), Animal reproduction. Principles and practices, McGraw-Hill Book Company, New York.

Sowers, M. R. and M. La Pietra (1995), Menopause: its epidemiology and potential association with chronic diseases, Epidemiol. Rev., 17(2), 287-302.

Swaisgood, R. R., D. M. Dickman, and A. M. White (2006), A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females, Biological Conservation, 129(4), 468-476.

Tubbs, C. (2012), Activation of Southern White Rhinoceros (*Ceratotherium simum simum*) Estrogen Receptors by Phytoestrogens: Potential Role in the Reproductive Failure of Captive-Born Females? Endocrinology, 153(3), 1444-1452.

Versteege, L. (2012), European Studbook White Rhino (*Ceratotherium simum simum*). White Rhinoceros European Endangered Species Program. Annual Report 2012.

Walker, C. L. (2002), Role of hormonal and reproductive factors in the etiology and treatment of uterine leiomyoma, Recent Prog. Horm. Res., 57, 277-294.

Walzer, C. and F. Schwarzenberger (1995), Estrous induction in a white rhinoceros (*Ceratotherium simum simum*) and concomitant EIA fecal progestagen analysis, Paper presented at Proceedings of the American Association of Zoo Veterinarians.

White, A. M., R. R. Swaisgood, and N. Czekala (2007), Differential investment in sons and daughters: do white rhinoceros mothers favor sons?, Journal of Mammalogy, 88(3), 632-638.

Wildt, D. E., P. Comizzoli, B. Pukazhenthi, and N. Songsasen (2010), Lessons from biodiversity—the value of nontraditional species to advance reproductive science, conservation, and human health, Mol. Reprod. Dev., 77(5), 397-409.

Chapter 3

Faecal progestagen profiles during pregnancy in wild southern white rhinoceros (*Ceratotherium simum simum*)

3.1. Abstract

Knowledge of the reproductive biology of wild animals can provide valuable information for the development of appropriate *in situ* and *ex situ* management plans. The present study aimed to establish a non-invasive protocol for monitoring faecal progestagen metabolite (FPM) patterns in wild female southern white rhinoceroses (*Ceratotherium simum simum*). Six adult females at Lapalala Wilderness Reserve, South Africa, were tracked and accurately identified at least once every week. Three animals gave birth during the study period. Fresh faecal samples were collected for 12 months and stored frozen at -20°C until analysis with an enzyme immunoassay utilising an antiserum raised against *5α*-pregnan-3*β*-ol-20-one which cross reacts with a number of progestagens. Mean FPM concentrations were 35 to 64-fold higher during pregnancy (55-145 days before parturition) compared to postpartum (120-140 days after parturition) ($p < 0.001$). Also, the non-pregnant animals had mean FPM concentrations significantly higher then postpartum values ($p = 0.006$). Our results show that non-invasive FPM measurements provide information on the pregnancy status of wild female white rhinoceroses, and may be used for the detection of pregnancy in

free-living individuals, without the necessity of immobilization and/or relocation of the animal. This information has potential value for optimizing breeding management of wild and captive populations.

3.2. Introduction

The African white rhinoceros (*Ceratotherium simum),* which was rescued from extinction at the end of the 19th century, is one of the five remaining species of rhinoceroses today. Together with the other surviving rhinoceros species: African black (*Diceros bicornis*), Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus)* and Sumatran (*Dicerorhinus sumatrensis*) (Owen-Smith 1992), the white rhinoceros population faces an uncertain future. One of the major causes is the extremely high demand for its horn, which is being used as an ingredient in traditional Asian medicine and also in the manufacture of ceremonial curved daggers in the Middle East (Owen-Smith 1992; Emslie *et al.* 2009). Horn poaching involves killing the animal and removal of its horns. Due to illegal hunting the black rhinoceros population, for example, has faced an overall population decline of 96% between 1970 and 1992 with a total number of 2,162 animals left at that time (Emslie *et al.* 2009). Illegal hunting in South Africa is again steadily increasing from around 210 black and white rhinoceroses being poached in total between 2006 and 2009 to 333 in 2010, reaching 448 in 2011 (Milliken *et al.* 2009; Knight 2011). According to the latest 2012 census, by the South African Department of Environmental Affairs, 668 rhinoceroses have been illegally killed in 2012

(DEA, 06/03/2013) indicating a worsening situation. Rhinoceros horn has been valued for centuries by Asian traditional healers to cure a variety of ailments, such as snakebites, fever, headaches and food poisoning. However, one of the reasons described for the rapid increase in recent poaching accidents is the increase in demand for new use of rhinoceros horn in modern Asian therapies to treat diseases such as cancer, rheumatism and gout (Costa-Neto 2004; Milliken *et al.* 2009). Two genetically distinct subspecies of white rhinoceros exist, namely the northern white rhinoceros (*Ceratotherium simum cottoni*) and the southern white rhinoceros (*Ceratotherium simum simum*) (Owen-Smith 1992). Ground surveys in 2009 suggested that the northern white rhinoceros was most likely eliminated from its range by civil war and poaching, with only 8 individuals remaining of this subspecies (Emslie *et al.* 2009) and in December 2009 4 captive individuals were re-introduced from Dvur Kralove Zoo into Ol Pejeta Conservancy, into a secure reserve in Kenya, in an attempt to encourage natural breeding in the wild (Milliken *et al.* 2009). The southern white rhinoceros population is currently estimated to be 20,920 (Emslie 2011) of which 93% live in South Africa (Milliken *et al.* 2009). Although this number seems viable, rapid decimation of the population is possible, especially given the recent steady increase in poaching incidents (Ferreira 2012). Being classified as “Near Threatened” on the IUCN’s Red List of Threatened Species (Emslie 2011), the southern white rhinoceros population is dependent on effective protection and intensive conservation and management (Amin *et al.* 2003; Hermes *et al.* 2005). If the situation arose where the population became vulnerable again – as observed a century ago

with only approximately 200 individuals remaining in the wild (Rookmaaker 2001) – successful breeding becomes an indispensable component of the overall conservation management strategies of the species.

Ex situ breeding can play a key role in maintaining rhinoceros populations today, with these captive populations serving as genetic stock and potential reservoirs for reintroduction into the wild. However, white rhinoceros females in captivity, especially from the captive-born (F1) generation onwards, show low reproductive success and aberrant cycling patterns. With a negative growth rate in captivity (-3.5% as a percentage of the entire captive population), the sustainability of the captive population may be jeopardized (Roth 2006; Swaisgood *et al.* 2006; AZA 2009). A recent study found death rate to have been 1.19 times higher than birth rate for 2001-2004 (Reid *et al.* 2012). Currently, the captive white rhinoceros population is static ($\lambda = 1.001$) only because of the continuous importation of new individuals from the wild (Foose & Wiese 2006; Emslie 2011). Thus, the current captive population is not self-sustainable. In contrast, observational studies suggest that wild white rhinoceroses do not show reproductive impairment (Swaisgood *et al.* 2006).

Our current understanding of the reproductive biology of the southern white rhinoceros is still limited. Literature reports the existence of two different oestrous cycle lengths in captive white rhinoceros, one with a luteal phase length of 30-35days and the other of 65-70days. Only the shorter cycle is believed to be fertile (Hindle *et al.* 1992; Schwarzenberger *et al.* 1993; Radcliffe *et al.* 1997; Patton *et al.* 1999; Brown *et al.* 2001).

Also, periods of acyclicity have been found in captivity with a high incidence in both young and aged females (Brown *et al.* 2001; Hermes *et al.* 2006). These repeated non-fertile periods are potentially contributing to the development of a variety of reproductive pathologies (Hermes *et al.* 2006). The length of gestation in the white rhinoceros is estimated to be 16 – 17 months (Patton *et al.* 1999). Pregnancy can be diagnosed approximately 3-5 months post conception by elevated progesterone concentrations (Roth 2006). Only a few observations have been made related to female reproduction in the wild (Owen-Smith 1992; Bertschinger 1994) and to date, no long-term reproductive hormone data are available from wild individuals. Hormones are often measured in blood samples, and it is possible to condition captive individuals to collect routine blood samples without the use of anaesthesia (Steele 2002). In contrast, collecting regular blood samples from wild rhinoceroses is impractical, as the animal would need to be sedated, captured and intensively monitored by an anaesthetic team for each single blood sample, thus only opportunistic blood sample collections is justified when accompanying essential immobilisation for other purposes. With the development of faecal hormone assays, it is possible to collect samples in a non-invasive way, and these methods have been used to study reproduction in captive held rhinoceroses (Radcliffe *et al.* 1997; Schwarzenberger *et al.* 1998; Patton *et al.* 1999; Brown *et al.* 2001). Apart from the practical advantages, this technique also bypasses the potential negative effects of stress on the results when using invasive methods (Christensen *et al.* 2006; Wittemyer *et al.* 2007). Investigating reproductive hormone

profiles will increase our knowledge of the reproductive physiology of wild female white rhinoceroses, and may contribute to understanding reproductive impairment in captivity.

3.2.1. Aims and strategy

The aim of the current study was to provide longitudinal data on faecal progestagen metabolite (fPM) concentrations in pregnant and non-pregnant wild individuals.

3.3. Material and methods

3.3.1. Animals and study site

Fieldwork was conducted for a period of 25 months between October 2008 and October 2010 in the Lapalala Wilderness, a 36,000 ha privately owned game reserve in the UNESCO Waterberg Biosphere Reserve in the Limpopo province, South Africa (23◦ 51 S, 28◦ 16 E). Lapalala Wilderness is situated in a summer rainfall area, with an annual rainfall ranging from 650 to 900 mm (Low & Rebelo 1996). Average maximum summer and winter temperature is 32 ◦C and 22 ◦C, respectively, and average minimum temperature is 18 ◦C for summer and 4 ◦C for winter (Ben-Shahar 1987). The vegetation in Lapalala Wilderness belongs to the savanna biome and is classified as Waterberg moist mountain bushveld (Low & Rebelo 1996).

Southern white as well as black rhinoceros have been successfully re-introduced into the reserve since the 1990s (Walker 1994). Based on information on previous reproductive success (Lapalala Wilderness, unpublished data), six adult female Southern white rhinoceroses were chosen as focal

individuals for the study (Table 3-1). Three females gave birth during the course of this study. The date of birth of the focal individuals was estimated based on recorded ground observations by qualified rangers (Lapalala Wilderness, unpublished data). Existing information on individual inter-birth intervals as well as the estimated age of the youngest calf (Table 3-1) was used for estimating the state of reproduction of the focal individuals.

Study animal	*Date of birth (est.)*	*No. of calves*	*Estimated age youngest calf*	*Mean interbirth interval*
Female 1	01 Dec 1993	4	1 yrs 7 months	2 yrs 7 months
Female 2	01 Apr 1993	3	2 yrs 8 months	2 yrs 11 months
Female 3	01 Jan 1993	3	1 yrs 8 months	2 yrs 5 months
Female 4	01 Feb 1996	2	4 yrs 11 months	2 yrs 9 months
Female 5	01 Jan 1989	5	1 yrs 2 months	3 yrs 5 months
Female 6	04 Nov 2003	0	-	-

Table 3-1. Background information of the white rhinoceros individuals (n=6) used for this study, including the estimated age of accompanying (youngest) calf at onset of project, i.e. October 2008. The youngest calf of female 4 died a few hours after birth on 01/07/2006 for unknown reasons, therefore her older calf born on 04/11/2003 (i.e. Female 6) was never chased away permanently. Female 6 was nulliparous at onset of project.

3.3.2. Animal tracking and identification

The location of the animals when they were out of sight was determined by tracking their spoor and by predicting their position based on observed trends in individual movement patterns. Discovered tracks were assessed on size (adult, sub adult or calf) and accompaniment of a calf or other individuals and followed on foot or by vehicle until the individual was visible. Animals were subsequently identified using physical characteristics such as ear notches (Fig. 3-1), horn size and scar tissue (Emslie & Brooks 1999). To avoid disturbance of the study animals and other group members, animals were observed from a distance against the wind direction, and noise and movement were kept to a minimum. The name of the area, GPS coordinates and the accompaniment of other individuals were documented each time an individual was localized.

Figure 3-1. Ear notching system used in white rhinoceros for individual identification.

3.3.3. Faecal sample collection

Regular faecal samples were collected from six wild adult female southern white rhinoceroses in a nature reserve in South Africa (Fig. 3-2). Each focal animal was observed at least at weekly frequency during the entire study period for faecal collection. Faecal samples were collected within 30 min after sighted defecation. When defecation was not observed, for example when the female defecated behind bushes or when fresh faeces were found nearby and on her track, this was noted. When the group existed of more than one adult female and defecation was not sighted, making it impossible to differentiate, no faecal sample was collected (Schenkel et al. 1969). Approximately 50g of homogenized faecal material was collected with rubber gloves from the inner part of a fresh faecal dropping and transferred directly into a pencil-marked conical 30 mL plastic vial. All samples were placed on ice immediately and stored within two hours of collection at -20°C to avoid influence of environmental factors on the steroid concentration (Washburn & Millspaugh 2002) until analysis at the Endocrine Research Laboratory, University of Pretoria, South Africa.

3.3.4. Sample processing and extraction

Faecal samples were lyophilized, pulverized, and sifted using a metal mesh strainer to remove fibrous material (Fieß et al. 1999). Approximately 0.05 g of the faecal powder was then extracted with 80% ethanol in water (3ml) by vortexing for 15 min and subsequent centrifugation for 10 min at 1500g. Resulting supernatants were transferred into micro-centrifuge tubes and stored at -20 °C until analysis (Appendix 1).

3.3.5. Assay procedures

Faecal extracts were measured for immunoreactive progesterone metabolites using an enzyme immunoassay (EIA) for 5α-pregnan-3β-

Figure 3-2. Southern white rhinoceros, female 2, with a 4-day-old newborn calf in Lapalala Wilderness, South Africa.

ol-20-one, which has been shown to provide reliable information on reproductive steroid hormone pattern by reflecting total progestagens in different mammalian species (Szdzuy et al. 2006; Ahlers et al. 2012). The EIA used a polyclonal antibody against 5α-pregnan-3β-ol-20-one-3-hemisuccinate-BSA and 5α-pregnan-3β-ol-20-one-3-hemisuccinate-peroxidase label (Szdzuy et al. 2006). Cross-reactivities of the antibody used are described by Szdzuy et al. (2006) and were as follows: 5α-pregnan-3α-ol-20-one, 650%; 5α-pregnan-3β-ol-20-one, 100%; 4-pregnen-3,20-dione, 72%; 5α- pregnan-3,20-dione, 22%; <0,1% for 5β-pregnan-3α,20α-diol,

4-pregnen-20α-ol-3-one, 5β-pregnan-3α-ol-20-one, 5α-pregnan-20α-ol-3-one, 5α-pregnan-3β,20α-diol and 5α-pregnan- 3α,20α-diol (Appendix 2). EIAs were performed following Prakash et al. (1987) (Appendix 3). Sensitivity (90% binding) of the assay was 4 pg/well. Intra- and interassay coefficients of variation, determined by repeated measurements of high and low value quality controls ranged between 9.3% and 16.5%. To adjust for water content variations, fPM concentrations were expressed as mass/dry mass of faecal extract.

3.3.6. Data analysis

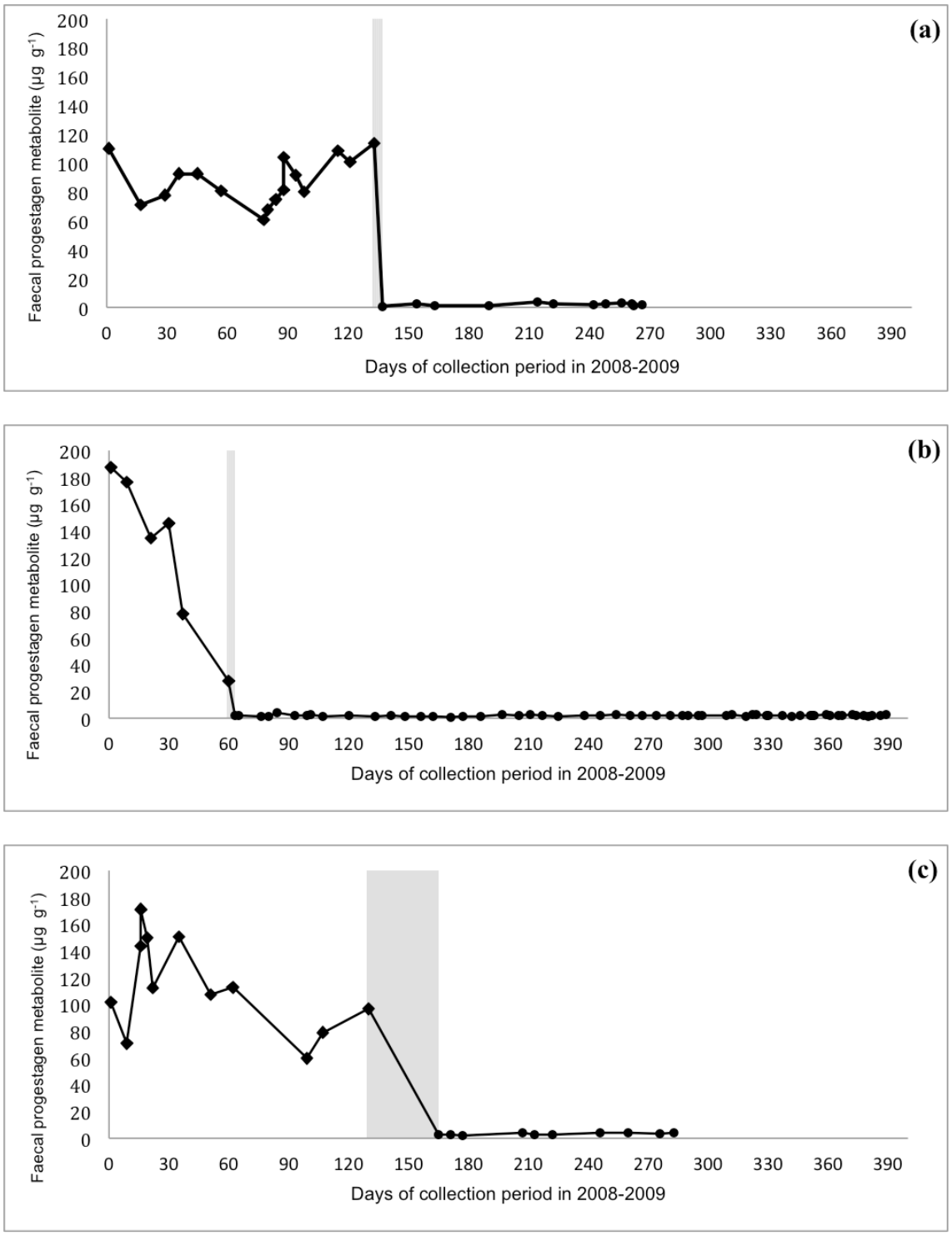
We allocated the faecal endocrine data into three different reproductive states; 1) pregnant (days 55 – 145 prior to parturition), 2) postpartum (days 120-140 following parturition), and 3) non-pregnant (cycling and/or anoestrus). We evaluated the effect of reproductive state on mean progestagen metabolite concentrations using a mixed model. This model contained the reproductive state as a fixed categorical predictor and sample date nested within each individual as a random effect structure. We fitted a variance power function to account for heteroscedasticity. We evaluated the fixed effect by a conditional F-test (Pinheiro & Bates 2000) and pair wise comparisons of reproductive states using Tukey contrasts. Statistical analyses were conducted using the package R version 2.15.1 for linux (http://www.r-project.org). We have reported data as individual means ± 1SD.

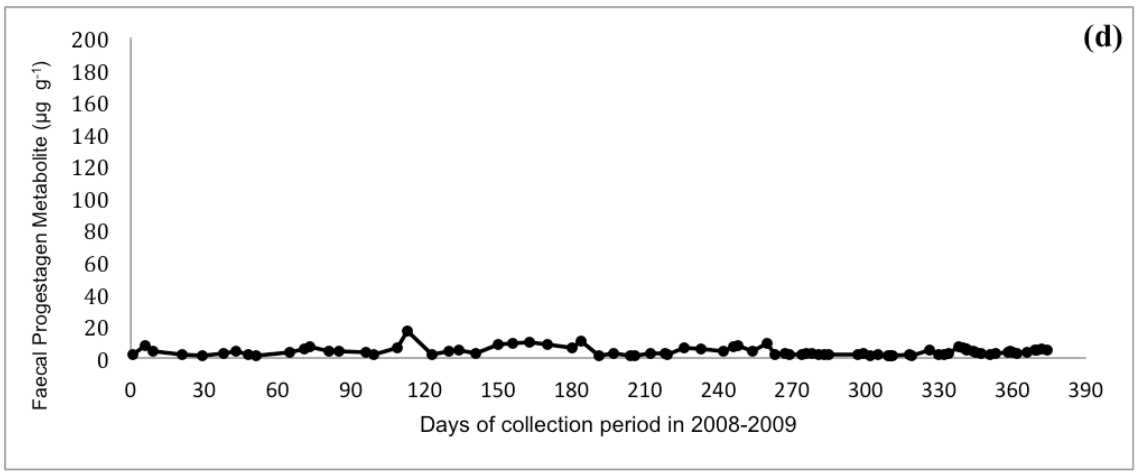
3.4. Results

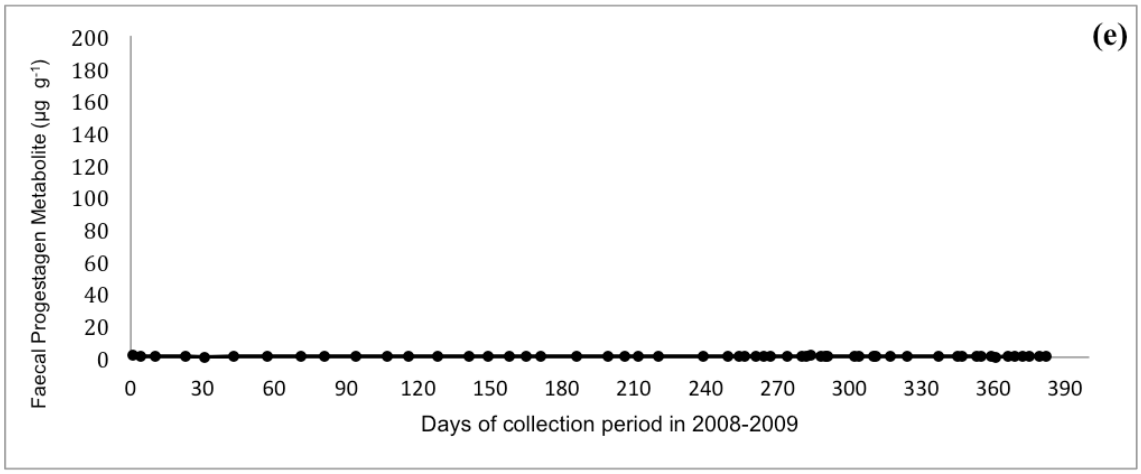
There was a significant effect of reproductive state on

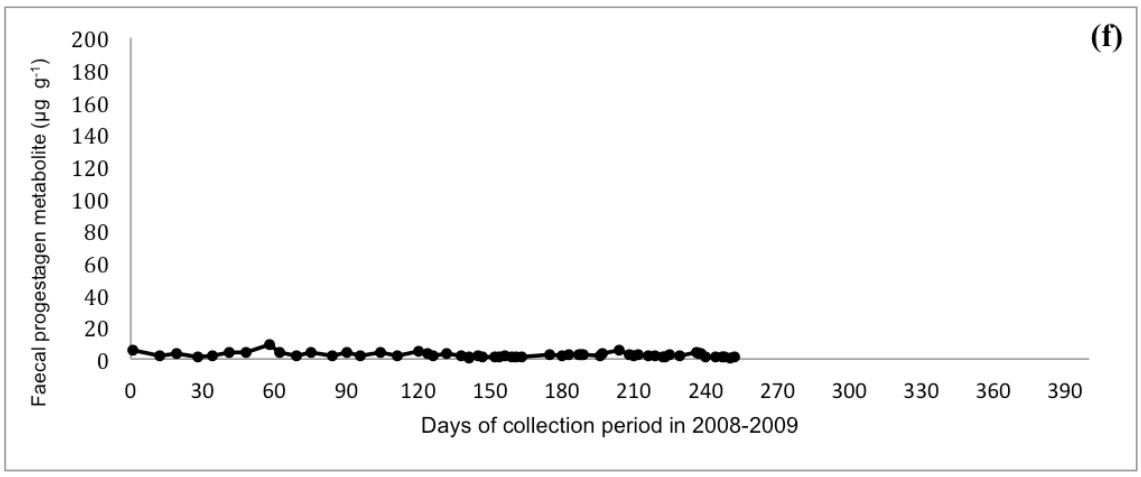
progestagen metabolite concentrations ($F_{2,308}$ = 49.54, p < 0.001), with mean fPM concentrations during the pregnant phase being

Figure 3-3. Concentrations of the faecal progestagen metabolite 5α-pregnan-3β- ol-20-one in wild southern white rhinoceros female 1 (a), female 2 (b) and female 3 (c) during late pregnancy (◆) and postpartum (•) and in non-pregnant wild southern white rhinoceros female 4 (d), female 5 (e) and female 6 (f). Moment of parturition (grey area) was estimated based on the visual observation of a new calf.









significantly higher than both the postpartum ($z = 9.47$, $p < 0.001$) and the non-pregnant ($z = 9.51$, $p < 0.001$) phase. Faecal concentrations in the non-pregnant phase were also significantly higher than the postpartum phase ($z = 2.56$, $p = 0.006$). All three pregnant females showed a rapid decline in fPM

concentrations around parturition and mean fPM concentrations prior to parturition were 35-64 fold higher (124.87 ± 61.16 µg/g faeces, 88.03 ± 16.19 µg/g faeces, and 112.86 ± 34.72 µg/g of faeces; Fig. 3-3 a,b,c) than concentrations found postpartum (3.09 ± 6.02 µg/g faeces, 2.11 ± 0.88 µg/g faeces, 3.20 ± 0.83 µg/g faeces, respectively). Concentrations remained in this range for the following 120-140 days of postpartum sample collection in all three females.

Although mean progestagen metabolite concentrations for non-pregnant females were significantly higher then the postpartum values, the differences were several orders of magnitude lower than the differences to the pregnant phase (4.05 ± 2.63 µg/g faeces, 2.54 ± 1.42 µg/g faeces, 0.58 ± 0.22 µg/g faeces; Fig. 3-3 d,e,f). In one of the three non-pregnant females (Fig. 3-3 d) attempted mounting behaviour was observed (no observed penetration) during the course of the study.

3.5. Discussion

Knowledge of the reproductive biology of wild animals can provide valuable information for the development of appropriate *in situ* and *ex situ* management plans. This paper provides the first description of progestagen metabolite profiles of non-pregnant wild white rhinoceroses ranging freely in their natural habitat as well as individual females during the latter stages of pregnancy, parturition and during the postpartum period.

Mean FPM concentrations in all pregnant females were substantially higher during pregnancy than postpartum and non-pregnant status. This technique thus facilitates the detection

of pregnancy in wild individuals in a non-invasive way, which can also be useful for surveillance strategies and the detection of foetal death during pregnancy. The mean FPM profiles in wild southern white rhinoceroses found in this study resemble those found in captive white rhinoceros individuals (Schwarzenberger *et al.* 1998; Patton *et al.* 1999). The number of samples collected for this study was insufficient to detect ovarian cycling patterns based on and pinpoint oestrus in the non-pregnant individuals. It would therefore be necessary to conduct a study equivalent to this one, focusing on more regular long term monitoring of FPM patterns in wild non-pregnant individuals. Such an approach could be subsequently used to compare the aberrant patterns seen in the captive white rhinoceros with patterns present in successfully reproducing free-living individuals (Schwarzenberger *et al.* 1998; Swaisgood *et al.* 2006).

In two of the three monitored pregnant females a suggestion of a decline of progestagen metabolite concentration prior to parturition was observed (Fig. 3-3 b,c). A previous study describing 5α-reduced pregnane profiles in captive white rhinoceroses did not observe this decline prior to parturition (Patton *et al.* 1999). However, one study found a decline in FPM during the last two weeks of pregnancy in the captive black rhinoceros (Schwarzenberger *et al.* 1993), although this could also be related to the specificity of the antibody used. More detailed endocrine data from white rhinoceroses around parturition are needed to reach a consensus conclusion as to whether a decline of FPM concentrations is, or is not, present in the wild white rhinoceros prior to and/or after parturition.

The data collected 120-140 days following parturition seems to show a consistent postpartum period of reproductive inactivity of at least 120 days in all three females. This observation has been described for white (Schwarzenberger *et al.* 1999) and Indian (Rietschel 2000) rhinoceroses in captivity as well as black rhinoceroses in the wild (Garnier *et al.* 2002).
As a potential alternative approach to progestagen measurement, ovarian characteristics and pregnancy could be monitored by measuring faecal oestrogen metabolites. An appropriate assay has to be carefully validated in terms of applicability for the species-specific hormone matrix of interest to ensure reliable hormone quantification (Hodges *et al.* 2010), and so far, no test system exist for a reliable measurement of oestrogen metabolites in white rhinoceros faeces. If studies like these in wild individuals can be expanded, through more frequent faecal collection and/or the validation of a faecal oestrogen metabolite assay, as demonstrated for the Indian rhinoceros (Stoops *et al.* 2004), data derived from such studies will provide us with new information on the reproductive physiology of the white rhinoceros, which could help considerably in diagnosing, understanding and solving the current breeding problem seen in the southern white rhinoceros in captivity. Furthermore, new insights into captive management related factors contributing to the reproductive failure observed in the southern white rhinoceros subspecies, could help us finding strategies to understand similar observations of reduced fertility in the critically endangered northern white rhinoceros subspecies.

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3.7. References

AHLERS, M.J., GANSWINDT, A., MÜNSCHER, S. & BERTSCHINGER, H.J. 2012. Fecal 20-oxo-pregnane concentrations in free-ranging African elephants (*Loxodonta africana*) treated with porcine zona pellucida vaccine. *Theriogenology* 78: 77-85.

AMIN, R., BRAMER, M. & EMSLIE, R. 2003. Intelligent data analysis for conservation: Experiments with rhino horn fingerprint identification. *Knowledge-Based Systems* 16: 329-336.

AZA 2009. Rhino Research Advisory Group: Five-year Research Master Plan for Rhinoceros. American Zoo and Aquarium Association.

BEN-SHAHAR, R. 1987. Grasses and habitat relationships on a sour bushveld nature reserve. *Vegetation* 72: 45–49.

BERTSCHINGER, H. J. 1994. Reproduction in black and white rhinos: A review. *Proceedings of a Symposium on Rhinos as Game Ranch Animals*, 96-99. Onderstepoort, Republic of South Africa, 9-10 September 1994.

BROWN, J. L., BELLEM, A. C., FOURAKER, M., WILDT, D. E. & ROTH, T. L. 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. *Zoo Biology* 20: 463-486.

CHRISTENSEN, B. W., TROEDSSON, M., LITTLE, R., OLIVA, M. & PENFOLD, L. 2006. Invasive and non-invasive methods of hormone monitoring in captive wildlife. *Proceedings of the Fourth Rhino Keepers Workshop 2005,* Columbus, Ohio.

COSTA-NETO, E. 2004. Implications and applications of folk zootherapy in the state of Bahia, northeastern Brazil. *Sustainable Development* 12: 161-174.

EMSLIE, R. 2011. *Ceratotherium simum ssp. simum*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. <www.iucnredlist.org>. Downloaded on 28 November 2012.

EMSLIE, R. H., AMIN, R. & KOCK, R. 2009. Guidelines for the in situ re-introduction and translocation of African and Asian rhinoceros. *Occasional Paper of the IUCN Species Survival Commission* 39: 16.

EMSLIE, R. H. & BROOKS, M. 1999. *African rhino: Status survey and conservation action plan*. IUCN/SSC African Rhino Specialist Group. Gland, Switzerland and Cambridge, UK.

FERREIRA, S.M., BOTHA, J.M. & EMMETT, M.C. 2012. Anthropogenic influences on conservation values of white rhinoceros. PLoS ONE 7: e45989.

FIEß, M., HEISTERMANN, M. & HODGES, J.K. 1999. Patterns of urinary and fecal steroid excretion during the ovarian cycle and pregnancy in the African elephant (*Loxodonta africana*). *General and Comparative Endocrinology* 115: 76–89.

FOOSE, T. J. & WIESE, R. J. 2006. Population management of rhinoceros in captivity. *International Zoo Yearbook* 40: 174-196.

GARNIER, J., HOLT, W., & WATSON, P. 2002. Non-invasive assessment of oestrous cycles and evaluation of reproductive

seasonality in the female wild black rhinoceros (*Diceros bicornis minor*). *Reproduction* 123: 877-889.

HERMES, R., HILDEBRANDT, T. B., BLOTTNER, S., WALZER, C., SILINSKI, S. & PATTON, M. L. 2005. Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*): Evaluation of male genital tract morphology and semen quality before and after cryopreservation. *Theriogenology* 63: 219-238.

HERMES, R., HILDEBRANDT, T. B., WALZER, C., GÖRITZ, F., PATTON, M. L., SILINSKI, S., ANDERSON, M.J., REID, C.E., WIBBELT, G., TOMASOVA, K. & SCHWARZENBERGER, F. 2006. The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*). *Theriogenology* 65: 1492-1515.

HINDLE, J. E., MOST, E. & HODGES, J. K. 1992. Measurement of urinary oestrogens and 20{alpha}-dihydroprogesterone during ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. *Journal of Reproduction and Fertility* 94: 237-249.

HODGES, K., BROWN, J. & HEISTERMANN, M. 2010. Endocrine monitoring of reproduction and stress. In: *Wild mammals in captivity: principles and techniques for zoo management,* (eds) D.G. Kleiman, K.V. Thompson & C. Kirk Baer, 447-468. University of Chicago Press, Chicago.

KNIGHT, M. 2011. *African Rhino Specialist Group report. Pachyderm* 49*:* 6-15.

MILLIKEN, T., EMSLIE, R. H. & TALUKDAR, B. 2009. *African and Asian rhinoceroses – status, conservation and trade.* A report from the IUCN species survival commission (IUNC/SSC) African and Asian rhino specialist groups and TRAFFIC to the CITES secretariat. The Hague, November 2009.

LOW, A. B. & REBELO, T.G. 1996. *Vegetation of South Africa, Lesotho and Swaziland*. Department of Environmental Affairs and Tourism, Pretoria, South Africa.

OWEN-SMITH, R. N. 1992. *Megaherbivores: The influence of very large body size on ecology*. Cambridge University Press.

PATTON, M. L., SWAISGOOD, R. R., CZEKALA, N. M., WHITE, A. M., FETTER, G. A. & MONTAGNE, J. P. 1999. Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. *Zoo Biology* 18: 111-127.

PINHEIRO, J.C. & BATES, D.M. 2000. Mixed effect models in S and S-plus. Springer Verlag, New York.

RADCLIFFE, R. W., CZEKALA, N. M. & OSOFSKY, S. A. 1997. Combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): Preliminary results. *Zoo Biology* 16: 445-456.

REID, C. E., VERSTEEGE, L., LUDWIG, A. & HERMES, R. 2012. Analysis of the European captive southern white rhinoceros (*Ceratotherium simum simum*) population using the International Studbook for the African White Rhinoceros (2001-2004) and integrating reproductive health findings. *International Zoo Yearbook* 46: 209-220.

RIETSCHEL, W. 2000. Case report and veterinary aspects of breeding the Indian rhino (*Rhinoceros unicornis*) at Stuttgart Zoo. In: *Proceedings of third meeting of the European Association of Zoo and Wildlife Veterinarians*, 195–200, Paris.

ROOKMAAKER, K. 2001. The alleged population reduction of the southern white rhinoceros (*Ceratotherium simum simum*) and the successful recovery. *Saugetierkundliche Mitteilungen* 45: 55-70.

ROTH, T. L. 2006. A review of the reproductive physiology of rhinoceros species in captivity. *International Zoo Yearbook* 40: 130-143.

SCHENKEL, R., SCHENKEL-HULLIGER, L. & HULLIGER, L.S. 1969. Ecology and behaviour of the black rhinoceros (*Diceros bicornis L.*): a field study, vol. 5, Paul Parey.

SCHWARZENBERGER, F., FRANCKE, R. & GOLTENBOTH, R. 1993. Concentrations of fecal immunoreactive progestagen metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*). *Journal of Reproduction and Fertility* 98: 285-291.

SCHWARZENBERGER, F., WALZER, C., TOMASOVA, K., VAHALA, J., MEISTER, J. & GOODROWE, K. L. 1998. Fecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*). *Animal Reproduction Science* 53: 173-190.

SCHWARZENBERGER, F., WALZER, C., TOMASOVA, K., ZIMA, J., GÖRITZ, F. & HERMES, R. 1999. Can the problems associated with the low reproductive rate in captive white rhinoceroses

(Ceratotherium simum) be solved within the next 5 years? *Erkrankungen Der Zootiere* 39: 283-289.

SZDZUY, K., DEHNHARD, M., STRAUSS, K., EULENBERGER, K. & HOFER, H. 2006. Behavioural and endocrinological parameters of female African and Asian elephants. *International Zoo Yearbook* 40*:* 41-50.

STEELE, V. 2002. Blood Collection. *Proceedings of the [second] Rhino Keepers' Workshop,* 43-44.

STOOPS, M. A., PAIRAN, R. D. & ROTH, T. L. 2004. Follicular, endocrine and behavioural dynamics of the Indian rhinoceros (*Rhinoceros unicornis*) oestrous cycle. *Reproduction* 128: 843-856.

SWAISGOOD, R. R., DICKMAN, D. M. & WHITE, A. M. 2006. A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. *Biological Conservation* 129: 468-476.

WALKER, C.H. 1994. Black rhino on private land - the experience of Lapalala Wilderness, South Africa. *Pachyderm* 18: 44-45.

WASHBURN, B. E. & MILLSPAUGH, J. J. 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. *General and Comparative Endocrinology* 127: 217-222.

WITTEMYER, G., GANSWINDT, A. & HODGES, K. 2007. The impact of ecological variability on the reproductive endocrinology of wild female African elephants. *Hormones and Behavior* 51: 346-354.

Chapter 4

Profiling patterns of fecal 20-oxopregnane concentrations during ovarian cycles in free-ranging southern white rhinoceros (*Ceratotherium simum simum*)

4.1. Abstract

Unlike their wild counterparts, many white rhinoceros females in captivity fail to reproduce successfully such that current captive populations are not self-sustainable. The causes of the problem are poorly understood. Variation in cycle length and long periods of acyclicity are a characteristic of the majority of these non-reproducing females but it is unknown whether these characteristics are a feature of free-living females. This study therefore aimed to monitor cyclic activity in a wild population of southern white rhinoceros at Lapalala Wilderness, South Africa, by measuring the concentrations of immunoreactive fecal progestagen metabolites (fPM). Fresh fecal samples were collected every week for 20 months from five reproductively successful adult females. Reproductive events and group structural dynamics were also recorded and subsequently correlated with the fPM data. The baseline concentration of fPM was 0.69 ± 0.20 µg/g DW while concentrations during pregnancy were 30- to 400-fold higher. Two females exhibited estrous cycle lengths of 30.6 ± 7.7 days (n = 7) and, based on fPM data, gestation length in one female was 502 ± 3 days. Year-round

monitoring showed no clear evidence of seasonality in ovarian activity. During cyclic luteal activity females were often seen in the presence of a dominant bull. One female stopped cycling after removal of the local dominant bull and luteal activity only returned after a new bull was introduced. This is a novel observation, which suggests that ovulation in white rhinoceros females in the wild may be influenced by external stimuli from a male. These findings indicate that the irregular cyclicity reported for white rhinoceros housed in zoos and animal parks may result from conditions in captivity and account for reduced fertility.

4.2. Introduction

The white rhinoceros (*Ceratotherium simum*), once endemic to sub-Saharan Africa (Prothero and Schoch, 1989), is one of the five species of rhinoceros remaining today, and together with the black (*Diceros bicornis*), Indian (*Rhinoceros unicornis*), Sumatran (*Dicerorhinus sumatrensis*) and Javan (*Rhinoceros sondaicus)* rhinoceros, faces an uncertain future due to heavy poaching for their horns (Milliken et al., 2009; Owen-Smith, 1992). It has been estimated that rhinoceros poaching in Africa has grown by 39% per year between 2008 and 2013 and, if this rate continues, rhinoceros numbers in Africa will start to decline between 2015 and 2016 (Emslie and Knight, 2014). The southern white rhinoceros population is currently estimated to be over 20,000 with approximately 4% living in captivity (Emslie and Knight, 2014).

If poaching cannot be controlled, managed breeding may become an important contributor to the global conservation of white rhinoceros as self-sustaining captive populations, as well as semi-captive populations where the animals are being farmed on large fenced plots under intensive management, can provide a valuable genetic reservoir for reintroductions into the wild (Emslie et al., 2009; Milliken et al., 2009). Unfortunately, in most breeding facilities, reproductive rate is poor in the white rhinoceros (Reid et al., 2012; Swaisgood et al., 2006; Versteege, 2012) with less than 50% of females reproducing even once, and the captive-born generation showing even lower success rates than the wild-caught generation, resulting in a non-self-sustaining population that is declining annually by 3.5% (AZA, 2014; Swaisgood et al., 2006; Versteege, 2012).

The reasons for this failure to breed are unclear but do not seem to be due poor male mating behavior or sperm quality (Hermes et al., 2005; Swaisgood et al., 2006). Attention is thus turning to female physiological factors. There have been several studies of the reproductive cycle of captive females based on the measurement of progestagens or their fecal and urinary metabolites (Brown et al., 2001; Carlstead and Brown, 2005; Hindle et al., 1992; Patton et al., 1999; Schwarzenberger et al., 1998). The data revealed distinct variation in cycle length, with short (30-35 days) and long cycles (65-70 days) detected (Brown et al., 2001; Hindle et al., 1992; Patton et al., 1999; Radcliffe et al., 1997; Schwarzenberger et al., 1998) and one female even displayed both cycle types (Patton et al., 1999; Schwarzenberger et al., 1998). However, only the shorter cycles seem to lead to pregnancy (Brown et al., 2001;

Schwarzenberger et al., 1998). Furthermore, long periods of anovulation and acyclicity have been demonstrated for 60% of captive females (Brown et al., 2001; Hermes et al., 2012; Hermes et al., 2006; Patton et al., 1999; Schwarzenberger et al., 1998). It is feasible that these prolonged, non-fertile periods are contributing to the development of reproductive pathologies seen in aged females (Hermes et al., 2012; Hermes et al., 2006) In contrast, much less is known about ovarian activity and its sex hormone correlates for female white rhinoceroses in the wild. Behavioral observations suggest the length of the reproductive cycle is 28 days (Bertschinger, 1994; Skinner and Smithers, 1990). However, there are no physiological data to confirm this, presumably because individual long-term monitoring of physiological markers requiring repeated sampling is usually difficult to conduct in the wild. Thus, it remains unknown whether free-living females also show two different cycle lengths or long periods of acyclicity, as seen in captive individuals. This situation can be remedied with the recent development of a non-invasive technique for monitoring changes in fecal progestagen metabolites in wild female white rhinoceros (Goot van der et al., 2013). This technique should be able to elucidate the reproductive pattern and its sex hormone correlates in wild populations of white rhinoceros, and subsequently help to improve breeding strategies for both wild and captive populations.

4.2.1. Aims and strategy

The main objective of this study was to characterize the estrous cycle of wild female white rhinoceroses in a population that

lives in its natural habitat and that is reproductively successful. Specifically, the hypotheses tested were: 1) estrous cycles in wild females can be determined using non-invasive fPM, 2) females in the wild only exhibit the single 30-35 day cycle; 3) females in the wild show limited periods of acyclicity compared to females in captivity.

4.3. Material and methods

4.3.1. Animals and study site

Five adult female southern white rhinoceroses aged 15-25 years were monitored at Lapalala Wilderness, Limpopo, South Africa (23° 51’ S, 28° 16’ E) for a period of 14-19 months between August 2011 and March 2013. All of the females were proven breeders with no history of reproductive abnormalities, and were likely to go through a period of ovarian cyclicity between two pregnancies during the scope of this study. Females roamed freely in this 36,000 ha privately owned nature reserve in the UNESCO Waterberg Biosphere Reserve and received no supplementary feeding. Paramilitary anti-poaching units effectively patrolled the entire reserve day and night during the course of the study. The region in which Lapalala Wilderness is located has summer rainfall and the annual rainfall ranges from 650 to 900 mm (Low and Rebelo, 1996). Average maximum temperature is 22 °C in winter and 32 °C in summer, and average minimum temperature is 4 °C winter and 18 °C in summer (Ben-Shahar, 1987). Two perennial rivers run through the area and the habitat is defined as savanna biome within the classification of Waterberg moist mountain bushveld (Low and Rebelo, 1996). The study was approved

by the Animal Ethics and Experimentation Committee of the University of Western Australia (RA/3/600/006).

4.3.2. Fecal sample collection and behavioral observations

Between August 2011 and March 2013, a total of 308 fecal samples were collected from the five females. Each individual was located, identified and observed on a weekly basis using established methods for tracking and identification (Emslie and Brooks, 1999; Goot van der et al., 2013). Disturbance of the animals during observation was prevented to avoid a potential influence of stress on the steroid concentrations (Carlstead and Brown, 2005). Approximately 50 g of homogenized fresh feces was collected within 30 min after defecation, placed on ice immediately and, within 1 hour, stored at –20 °C until analysis at the Endocrine Research Laboratory, University of Pretoria (Washburn and Millspaugh, 2002). Throughout the observation period, physical signs and/or socio-sexual behavior indicating the potential occurrence of estrus, as well as suckling behavior as an indicator for lactation, were documented (Owen-Smith, 1975). In anticipation of a female defecating, observation periods would normally last from 30 minutes to 3 hours. During this period, the physical appearance and behavior of the individual was monitored carefully and signs of mounting, mating, presence of a bull, birth, nursing, suckling calf, swollen vulva and/or vaginal discharge were noted (Owen-Smith, 1975; Patton et al., 1999).

4.3.3. Steroid hormone extraction and enzyme immunoassay (EIA)

Frozen fecal samples were lyophilized, pulverized, and sieved through a nylon mesh to remove fibrous material (Fieß et al., 1999). Pulverized feces (0.05 – 0.055 g) were extracted by vortexing for 15 min with 3 mL of 80% ethanol. Following centrifugation for 10 min at 1500 *g*, supernatants were transferred into micro-centrifuge tubes and stored at –20 °C until assay.

Concentrations of immunoreactive fecal progestagen metabolites (fPM) were measured by enzyme-immunoassay (EIA) technique using an antibody raised against 5*α*-pregnan-3*β*-ol-20-one-3-hydroxysuccinyl-BSA and 5*α*-pregnan-3*β*-ol-20-one-3-hydroxy succinyl-peroxidase as label (Szdzuy et al., 2006) according to the procedure described by Ganswindt et al. (2002). This EIA has been validated for determining fPM concentrations in southern white rhinoceroses and has been shown to provide reliable information on reproductive steroid hormone pattern by reflecting total progestagens in the white rhinoceros and other mammalian species (Goot van der et al., 2013). Cross-reactivities of the antibody are described by Szdzuy et al (2006). Serial dilutions of fecal extracts gave displacement curves that were parallel to the standard curve. Intra- and inter-assay coefficients of variations, determined by repeated measurements of high- and low-concentration pooled samples, ranged between 9.3% and 16.5%. Sensitivity of the assay at 90% binding was 3 pg per well.

4.3.4. Data analysis

Individual baseline values for fPM concentration, in µg/g dry weight (DW), were calculated using an iterative elimination process as previously described (Brown et al. 1994). In brief; all fPM concentrations of an individual data set exceeding the mean +2 standard deviations (S.D.) were excluded, the average successively recalculated, and the elimination process repeated until no values exceeded the mean + 2 S.D. The remaining values yielded the baseline fPM concentrations for the animal under consideration.

Hormone profiles were evaluated on indication of ovarian cyclicity and elevations in fPM concentration were considered to indicate a luteal phase if at least two values exceeded baseline level for at least 2 consecutive weeks (Ahlers et al., 2012; Brown et al., 2001). The end of the luteal phase was defined as the first sample in which fPM concentrations returned to baseline level (Brown et al., 2001). Physical and/or behavioral signs of estrus were aligned with episodes of elevated fPM concentrations. Data are presented as mean ± SEM.

4.4. Results

During the course of the study, all five females were always seen in groups of 2-8 animals, with the local dominant bull periodically joining a group for a few days. Three of the five females gave birth during the study period while the other two were already pregnant at the beginning of the monitoring program. Figure 4-1 shows the individual longitudinal fPM profiles of the three females (Female 1, 2 and 3) that gave

birth during the 20-month study period or shortly thereafter (Fig. 4-1A,B,C). In the two comprehensively monitored pregnancies (female 1 and 2), 400-fold decreases in fPM concentrations preceded parturition (Fig. 4-1A,B) and, in both cases, a newborn calf was seen with the mother a few days later, confirming successful pregnancy and delivery of viable offspring. In both of these females, postpartum fPM concentrations fluctuated up to 6-fold above baseline values, suggesting luteal activity, but no cyclic pattern could be detected in the 11-12 months following parturition (Fig. 4-1A,B). During this period, calf nursing and suckling was frequently observed, suggesting that both females were lactating (Fig. 4-1A,B).

Individual fPM baseline concentrations ranged from 0.41 to 0.92 µg/g DW. Female 3 conceived during the study period and showed regular cycles of luteal activity with a mean inter-luteal cycle length of 31.5 ± 8.5 days (n = 4 cycles) prior to conception (Fig. 4-1C). Its fPM baseline concentration was 0.47µg/g DW but, during luteal activity, fPM concentrations increased to peaks values of up to 3.58 µg/g DW. No estrous behavior or mating was observed prior to conception, although she was often seen together with the dominant bull during her period of regular cyclic luteal activity. After conception, fPM concentrations stayed above baseline values, often exceeding luteal phase concentrations from the 3rd month of gestation onwards.

Female 4 and 5 did not reflect pregnancy and both had calves, aged 9 and 1 months respectively, at the beginning of the study. Luteal activity was detected in both animals with fPM

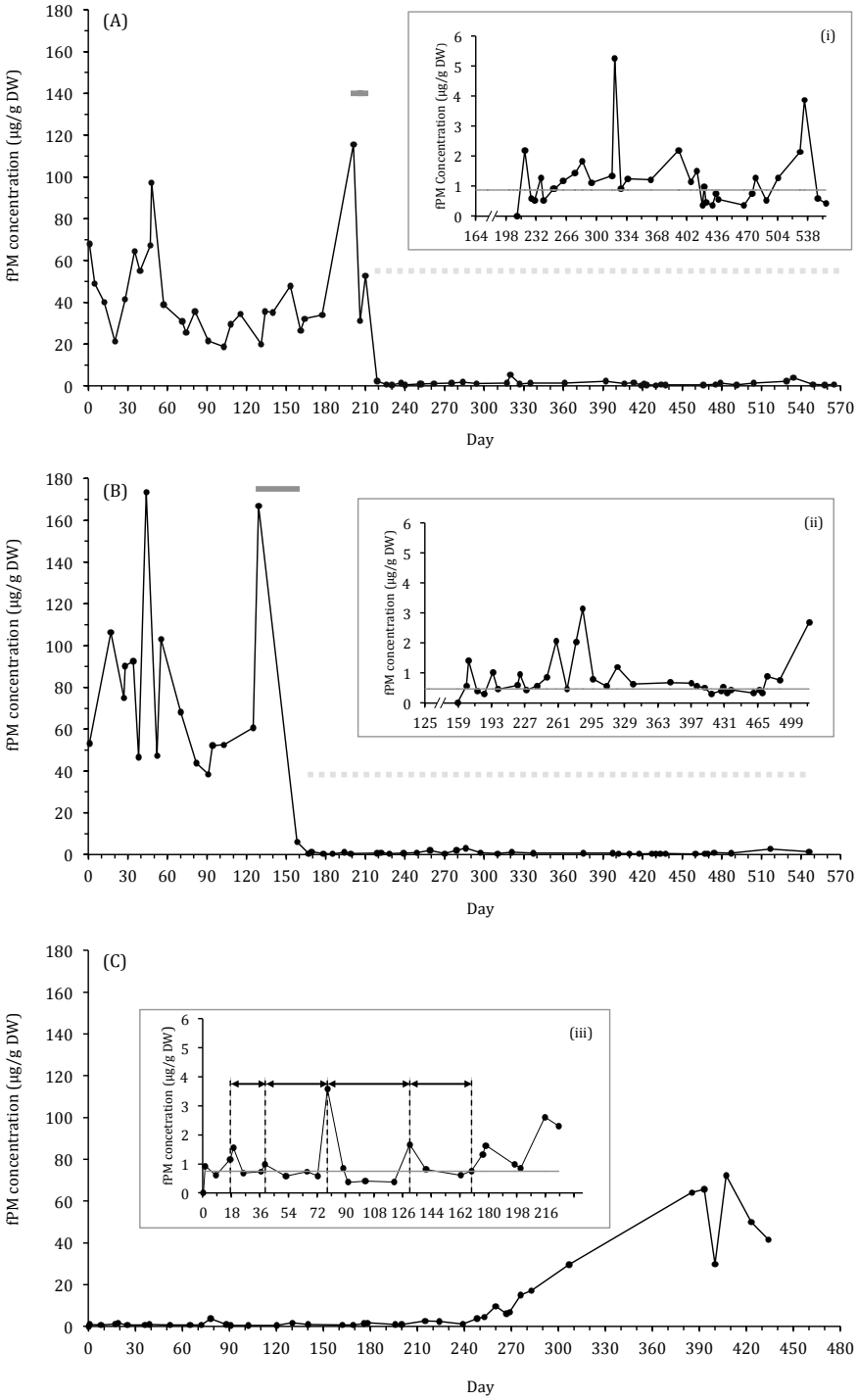

Figure 4-1. Longitudinal profiles of immunoreactive 5α-pregnan-3β- ol-20-one concentrations (●) for 3 adult female white rhinoceroses. Females 1 (A) and 2 (B) gave birth during the 20-month monitoring period. The time of parturition (grey bar) was estimated from the appearance of a new calf. The dotted horizontal line indicates lactation anestrus. Female 3 (C) gave birth shortly after the monitoring period in June 2013. Fecal PM concentrations during the non-pregnant periods are also shown with a different resolution in inserts (i, ii, iii), showing determined cyclic patterns (arrowed). The solid horizontal grey line in inserts indicates individually calculated baseline levels.

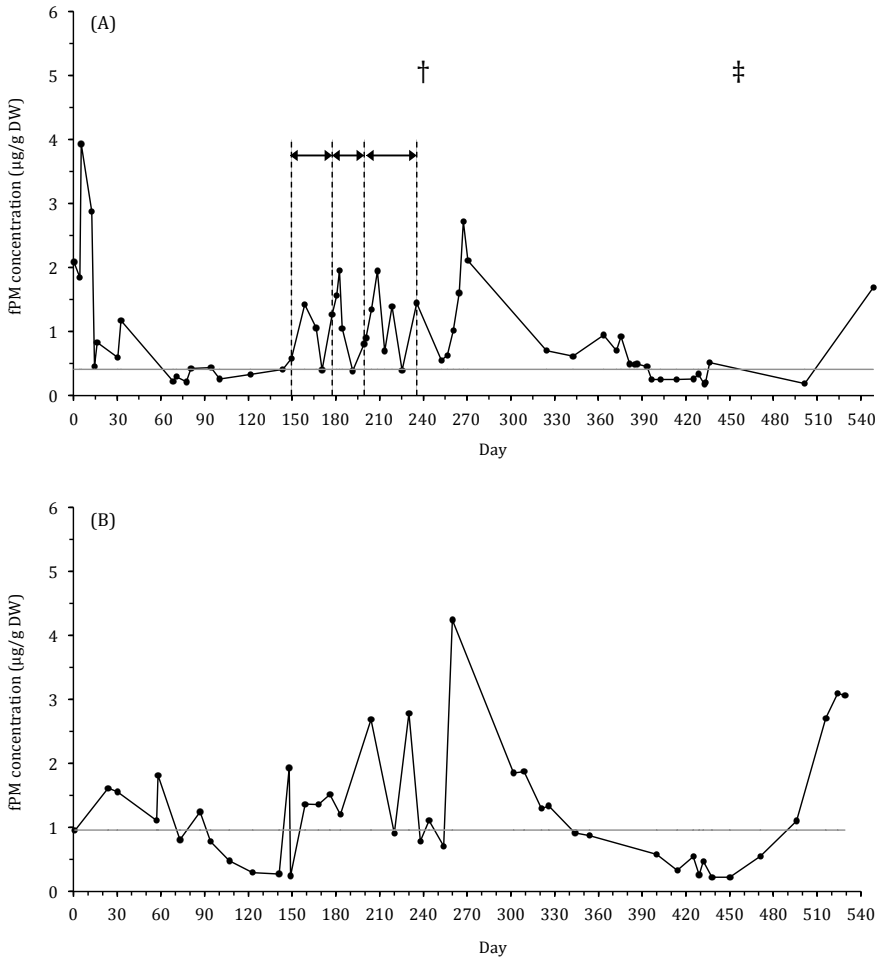

Figure 4-2. Longitudinal profiles of immunoreactive 5α-pregnan-3β- ol-20-one concentrations (●) in 2 adult female white rhinoceroses. Female 4 (A) and female 5 (B) were both accompanied by a young calf from the start of the monitoring period. Determined regular cyclic patterns are indicated (arrowed). The solid horizontal grey line indicates individually calculated baseline levels. The dominant bull in the home range of Female 4 was removed from the area (dagger) and a new bull was introduced (double dagger).

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concentrations reaching maximum values of 3.9 and 4.3 µg/g DW (Fig. 4-2A,B). For female 4 (Fig. 4-2A), regular cycles could not be detected for the first 5 months of data collection but, subsequently, regular cycles of luteal activity were evident for 85 days, with a mean inter-luteal cycle length of 29.3 ± 6.1 days (n = 3 cycles). During these cycles, fPM concentrations reached a maximum value of 3.9µg/g DW. No behavioral or physical signs of estrus or mating were observed during this time and,

towards the end of this period, the dominant bull of this female's home range was removed from the area. After the male was removed, fPM concentrations remained around baseline level. Seven months later, a new bull was introduced into the area, but the female moved to an extremely remote area in the reserve, minimizing sampling success. The female returned to its previous home range 5 months later and was observed mating with the newly introduced bull a month later. No regular cycles of luteal activity were detected in female 5 during the course of the study (Fig. 4-2B).

4.5. Discussion

This study is the first to characterize longitudinal profiles in the fecal concentrations of progesterone metabolites, thus allowing the description of ovarian activity in wild female white rhinoceros. We thus established that non-invasive fPM monitoring techniques can be used successfully in wild individuals to investigate ovarian cyclicity in this species, which is desirable due to inconsistency of the reproductive data for animals held in captivity. We have also established that it is feasible to collect samples on a regular basis from non-collared white rhinoceros females that roam freely in a 36,000 ha reserve. However, the success of sample collection did vary among focal animals in relation to accessibility of home range and individual temperaments, so feasibility trials should be carried out before monitoring females in different environments. A white rhinoceros can traverse large distances on a single day and, in the present study, the tracking of one individual often

took over 2 hours in a day. To increase success rate and frequency of sample collection, the use of advanced tracking technologies is recommended, especially when tracking in large areas.

A complete pregnancy was monitored in the present study, presenting the first longitudinal fPM profile of gestation in a wild white rhinoceros. The gestation length for this female was 500-505 days (~16.5 months), a figure that is consistent with previous estimates in the wild based on behavioral observations (480-547 days) (Bertschinger, 1994) as well as estimates for animals in captivity (490-525 days) (Patton et al., 1999). During gestation, fPM concentrations rose to be considerably higher than luteal phase concentrations by the 3rd month of gestation and peak concentrations were 30- to 400-fold higher than baseline values. Similar findings were reported by Schwarzenberger et al. (1998) who mentioned high fPM concentrations in pregnant captive females during the 4th and 5th months of pregnancy, as well as Hermes et al. (2012) who detected a clear increase 2 months after ovulation.

In this study, a total of 7 regular cycles were detected, with a mean cycle length (30.6 ± 7.7 days) that is consistent with lengths derived from behavioral observations (Skinner and Smithers, 1990). Neither of the two females that exhibited regular cycles had long cycles (65-70 days), which are commonly present in captive females (Brown et al., 2001; Patton et al., 1999; Schwarzenberger et al., 1998). It is feasible that long cycles reflect reproductive pathology and that females living in the wild without reproductive problems do not show this phenomenon. Diverse factors may be involved such as

phytoestrogens in captive diets high in alfalfa (Tubbs, 2012), stress of captivity (Carlstead and Brown, 2005) and absence of appropriate stimulation by a male rhino (Carlstead and Brown, 2005; Patton et al., 1999).

It is interesting that one female in the wild exhibited a long period of acyclicity that could not be attributed to lactation, as is commonly reported in captive females (Brown et al., 2001; Hermes et al., 2012; Hermes et al., 2006; Patton et al., 1999; Schwarzenberger et al., 1998). Lactation anestrus was visible in this study as an acyclic period until 13 months post partum, in accord with observational studies in the white rhinoceros (Brown et al., 2001; Owen-Smith, 1992; Schwarzenberger et al., 1993; Skinner and Smithers, 1990). Occasionally, concentrations fluctuated towards luteal levels during these periods but no regular cyclic activity was present. One female started displaying regular luteal activity when her calf was 13 months old, however, her regular luteal activity stopped suddenly and she then presented a long acyclic period. In the same month, the dominant bull was removed from the reserve for management purposes. It is unlikely that this observation was related to reproductive problems, because this female became pregnant shortly after a new adult male was introduced. These observations suggest that long periods of acyclicity are not uncommon in wild white rhinoceros and that ovulation might be influenced by the presence of a dominant male. Similar traits are known in species that do not ovulate spontaneously such as the domestic cat (*Felis catus*) and camelids (England et al., 1969; Novoa, 1970), the so-called “induced ovulators” that need mating or mounting to elicit a surge of luteinizing hormone (LH)

and thus ovulation (Wildt et al., 1981). However, in many induced ovulators, such as the leopard (*Panthera pardus*) and alpaca (*Lama pacos*), there is occasionally spontaneous ovulation without copulatory stimuli (England et al., 1969; Fernandez-Baca et al., 1970; Schmidt et al., 1988). In 2001, it was discovered that the Sumatran rhinoceros is an induced ovulator that commonly displays irregular cycling in the absence of mating (Roth et al., 2001). Although most induced ovulators are solitary species, the African lion is an example of an induced ovulator with highly structured social systems (Pusey and Packer, 1987). The white rhinoceros has never been considered an induced ovulator (Brown et al., 2001; Roth, 2006), but the present study suggests that external stimuli (contact with a dominant bull) might facilitate ovulation. Moreover, the occurrence of “long cycles” in captivity, perhaps due to luteinized follicles, could be explained, because luteinization of follicles is observed in the Sumatran rhinoceros and several other induced ovulators when ovulation does not occur (Roth et al., 2001). Further investigation, combining ultrasonography and progesterone monitoring, in both captive and wild populations, is necessary to establish whether the white rhinoceros is an induced ovulator or not.

An alternative explanation for the occurrence of a prolonged period of acyclicity that was not related to lactation could be seasonality in ovarian activity, as observed in equids (Brinsko et al., 2010). There is some evidence for reduced reproductive activity during the winter months for free-ranging nulliparous black rhinoceroses (Garnier et al., 2002) and, even in white rhinoceroses, season-related birth peaks have been recorded for

the end of the rainy season (Owen-Smith, 1992). However, captive rhinoceroses do not show any seasonal trend in reproduction (Schwarzenberger et al., 1998; Brown et al., 2001), suggesting that seasonal preferences for reproductive activity observed in free-ranging rhinoceroses might be more related to extrinsic factors like food availability (Owen-Smith, 1992) or photoperiod (Garnier et al., 2002; Brinsko et al., 2010).

In conclusion, the fPM data presented in this study on free-living white rhinoceros confirms the ‘short cycle’ of about 30 days as the normal length for ovarian cycles, and that gestation is about 500 days, as found in captive females. However, further studies with larger sample sizes and in different settings are needed to confirm these findings. While a lactation anestrus of about 13 months was confirmed in this study, a period of acyclicity not attributable to lactation was detected in a reproductively healthy free-living female, seemingly in association with the removal of a dominant bull, raising the possibility that the white rhinoceros is an induced ovulator. Further research that combines behavioral observations, ultrasonography and progesterone monitoring, is required to investigate social and environmental factors regulating cyclic activity in wild populations of white rhinoceros.

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4.7. References

Ahlers, M.J., Ganswindt, A., Münscher, S., Bertschinger, H.J., 2012. Fecal 20-oxo-pregnane concentrations in free-ranging African elephants (*Loxodonta africana*) treated with porcine zona pellucida vaccine. Theriogenology 78, 77-85.

AZA, 2014. Rhino Advisory Group's 2014 Research Masterplan. American Zoo and Aquarium Association.

Ben-Shahar, R., 1987. Grasses and habitat relationships on a sour bushveld nature reserve. Vegetatio 72, 45-49.

Bertschinger, H.J., 1994. Reproduction in black and white rhinos: a review. Proceedings of a symposium on rhinos as game ranch animals. Onderstepoort, Republic of South Africa, 9-10 September 1994, 96-99.

Brinsko, S.P., Blanchard, T.L., Varner, D.D., Schumacher, J., Love, C.C., 2010. Manual of equine reproduction. Elsevier Health Sciences.

Brown, J.L., Wasser, S.K., Wildt, D.E., Graham, L.H., 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biology of Reproduction 51, 776-786.

Brown, J.L., Bellem, A.C., Fouraker, M., Wildt, D.E., Roth, T.L., 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biol. 20, 463-486.

Carlstead, K., Brown, J.L., 2005. Relationships between patterns of Fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. Zoo Biol. 24, 215-232.

Emslie, R.H., Amin, R., Kock, R., 2009. Guidelines for the in situ re-introduction and translocation of African and Asian rhinoceros. Occasional paper of the IUCN Species Survival Commission 39, 16.

Emslie, R.H., Brooks, M., 1999. African rhino: Status survey and conservation action plan. IUCN/SSC African Rhino Specialist Group, Gland, Switzerland and Cambridge, UK.

Emslie, R.H., Knight, M.H., 2014. Update on African rhino status and trends: from IUCN SSC African Rhino Specialist Group (AfRSG), 1-6.

England, B.G., Foote, W.C., Matthews, D.H., Cardozo, A.G., Riera, S., 1969. Ovulation and corpus luteum function in the llama (*Lama glama*). J. Endocrinol. 45, 505-513.

Fernandez-Baca, S., Madden, D.H.L., Novea, C., 1970. Effect of different mating stimuli on induction of ovulation in the alpaca. Journal of Reproduction and Fertility 22, 261-267.

Fieß, M., Heistermann, M., Hodges, J.K., 1999. Patterns of urinary and fecal steroid excretion during the ovarian cycle and pregnancy in the African elephant (*Loxodonta africana*). Gen. Comp. Endocrinol. 115, 76-89.

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

Goot van der, A.C., Dalerum, F., Ganswindt, A., Martin, G.B., Millar, R.P., Paris, M.C.J., 2013. Faecal progestagen profiles in wild southern white rhinoceros (*Ceratotherium simum simum*). Afr. Zool. 48, 143-151.

Hermes, R., Hildebrandt, T.B., Walzer, C., Göritz, F., Gray, C., Niemuller, C., Schwarzenberger, F., 2012. Estrus induction in white rhinoceros (*Ceratotherium simum*). Theriogenology 78, 1217-1223.

Hermes, R., Hildebrandt, T.B., Blottner, S., Walzer, C., Silinski, S., Patton, M.L., Wibbelt, G., Schwarzenberger, F., Göritz, F., 2005. Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation. Theriogenology, 63, 219-238.

Hermes, R., Hildebrandt, T.B., Walzer, C., Göritz, F., Patton, M.L., Silinski, S., Anderson, M.J., Reid, C.E., Wibbelt, G., Tomasova, K., Schwarzenberger, F., 2006. The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*). Theriogenology 65, 1492-1515.

Hindle, J.E., Most, E., Hodges, J.K., 1992. Measurement of urinary oestrogens and 20{alpha}-dihydroprogesterone during ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. J. Reprod. Fertil. 94, 237-249.

Low, A.B., Rebelo, T.G., 1996. Vegetation of South Africa, Lesotho and Swaziland. Department of Environmental Affairs and Tourism, Pretoria, South Africa.

Milliken, T., Emslie, R.H., Talukdar, B., 2009. African and Asian rhinoceroses – status, conservation and trade. A report from the IUCN Species Survival Commission (IUNC/SSC) African and Asian Rhino Specialist Groups and TRAFFIC to the CITES Secretariat. CoP15 Doc. 45.1, 1-18.

Novoa, C., 1970. Reproduction in Camelidae. J. Reprod. Fertil. 22, 3-20.

Owen-Smith, R.N., 1992. Megaherbivores: the influence of very large body size on ecology. Cambridge University Press.

Owen-Smith, R.N., 1975. The Social Ethology of the White Rhinoceros Ceratotberium simum (Burchell 1817*). Zeitschrift Für Tierpsychologie 38, 337-384.

Patton, M.L., Swaisgood, R.R., Czekala, N.M., White, A.M., Fetter, G.A., Montagne, J.P., Rieches, R.G., Lance, V.A., 1999. Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. Zoo Biol. 18, 111-127.

Prothero, D.R., Schoch, R.M., 1989. The evolution of perissodactyls. Oxford University Press.

Pusey, A.E., Packer, C., 1987. The evolution of sex-biased dispersal in lions. Behaviour, 275-310.

Radcliffe, R.W., Czekala, N.M., Osofsky, S.A., 1997. Combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): Preliminary results. Zoo Biol. 16, 445-456.

Reid, C.E., Versteege, L., Ludwig, A., Hermes, R., 2012. Analysis of the European captive southern white rhinoceros Ceratotherium simum simum population using the International Studbook for the African white rhinoceros (2001-2004) and integrating reproductive health findings. International Zoo Yearbook.

Roth, T.L., 2006. A review of the reproductive physiology of rhinoceros species in captivity. International Zoo Yearbook 40, 130-143.

Roth, T., O'Brien, J., McRae, M., Bellem, A., Romo, S., Kroll, J., Brown, J., 2001. Ultrasound and endocrine evaluation of

the ovarian cycle and early pregnancy in the Sumatran rhinoceros, Dicerorhinus sumatrensis. Reproduction 121, 139-149.

Schmidt, A.M., Hess, D.L., Schmidt, M.J., Smith, R.C., Lewis, C.R., 1988. Serum concentrations of oestradiol and progesterone, and sexual behaviour during the normal oestrous cycle in the leopard (*Panthera pardus*). J. Reprod. Fertil. 82, 43-49.

Schwarzenberger, F., Francke, R., Goltenboth, R., 1993. Concentrations of faecal immunoreactive progestagen metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*). J. Reprod. Fertil. 98, 285-291.

Schwarzenberger, F., Walzer, C., Tomasova, K., Vahala, J., Meister, J., Goodrowe, K.L., Zima, J., Strauß, G., Lynch, M., 1998. Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*). Animal Reproduction Science 53, 173-190.

Skinner, J., Smithers, R. (Eds.), 1990. *The mammals of the southern African subregion.* University of Pretoria, Pretoria.

Swaisgood, R.R., Dickman, D.M., White, A.M., 2006. A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. Biological Conservation, 129, 468-476.

Szdzuy, K., Dehnhard, M., Strauss, G., Eulenberger, K., Hofer, H., 2006. Behavioural and endocrinological parameters of female African and Asian elephants *Loxodonta africana* and *Elephas maximus* in the peripartal period. International Zoo Yearbook 40, 41-50.

Tubbs, C., 2012. Activation of Southern White Rhinoceros (*Ceratotherium simum simum*) Estrogen Receptors by Phytoestrogens: Potential Role in the Reproductive Failure of Captive-Born Females? Endocrinology 153, 1444-1452.

Versteege, L., 2012. European Studbook White Rhino (*Ceratotherium simum simum*). White Rhinoceros European Endangered Species Program. Annual Report 2012.

Washburn, B.E., Millspaugh, J.J., 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. General and Comparative Endocrinology 127, 217-222.

Wildt, D.E., Chan, S.Y.W., Seager, S.W.J., Chakraborty, P.K., 1981. Ovarian Activity, Circulating Hormones, and Sexual Behavior in the Cat. I. Relationships During the Coitus-Induced Luteal Phase and the Estrous Period Without Mating. Biology of Reproduction 25, 15-28.

Chapter 5

Control of estrus and ovulation in white rhinoceros using a synthetic progestagen and slow-release GnRH analogue

5.1. Abstract

Breeding in captivity and semi-captive environments may play an important role in the survival of the conservation-dependent white rhinoceros (*Ceratotherium simum*). However, the overall reproductive rate in captivity is too low, and the captive population is not self-sustaining. One related factor is the inexplicable observation at many institutions of an aberrant cycling pattern in females. In order to increase breeding success in captivity, it is important to determine the direct causes of estrous cycle irregularity and also to develop an estrus and ovulation synchronization protocol that allows timed natural breeding, where ovulation is induced and followed by mating, and assisted reproduction. Several studies have involved an attempt to induce estrus in the white rhinoceros, but ovulation using those protocols was inconsistent from female to female. This study utilized a synthetic progestagen treatment followed by a slow-release GnRH analogue to synchronize estrus and ovulation among 3 southern white rhinoceroses. Oral synthetic progestagen (altrenogest, 0.022-0.44mg/kg/d) was administered for 21 days after a random start, followed by a single injection of a slow-release GnRH analogue (deslorelin

acetate, 2.5 µg/kg) 9.5 days after discontinuing progestagen treatment. Treatment success was determined by monitoring fecal progestagen metabolites using an enzyme immunoassay in daily fecal samples and behavioral observations were recorded on a daily basis. During the sample collection period, luteal activity was seen in all three females and in two females a synchronized luteal phase was detected. Interestingly, these observations did not seem to be related to our treatment protocol. One possibility is that the results could be associated with inaccurate test results and the use of an alternative enzyme immunoassay that successfully reflects progestagen concentrations in the white rhinoceros is necessary to confirm this hypothesis. This will then allow further research to improve the treatment protocol presented in this study, which could ultimately create a reliable protocol to induce ovulation during assisted breeding in the white rhinoceros.

5.2. Introduction

The African white rhinoceros (*Ceratotherium simum*) is one of the five remaining rhinoceros species which all face an uncertain future due to heavy poaching for their horns [Milliken et al., 2009]. Moreover, the captive population of white rhinoceros is currently being threatened by low reproductive success rates amongst the majority of breeding facilities [Swaisgood et al., 2006; Versteege, 2012; Reid et al., 2012]. Previous studies have indicated that less than 50% of white rhinoceros females in captivity reproduce successfully, with the captive-born (F1)

generation showing even lower success rates than the wild-caught (F0) generation [Swaisgood et al., 2006; Versteege, 2012]. This is in clear contrast with wild populations of white rhinoceros, which in general reproduce well with an average inter-calving period of 2.63-3.45 years [Owen-Smith, 1992; Bertschinger, 1994; Swaisgood et al., 2006; Goot et al., 2013]. Other observations amongst captive females that are currently being addressed are a high incidence of irregular cycling [T. L. Roth, 2006; Swaisgood et al., 2006], ovulatory failure [Hermes et al., 2005] and observed periods of acyclicity associated with pathologies of the reproductive tract [Brown et al., 2001; Hermes et al., 2004]. Possible contributing factors are likely to be management-related as facilities with larger enclosures and bigger group sizes seem to show improved pregnancy results [Metrione, 2010]. Many of the potential factors identified are yet to be further assessed.

Breeding in captivity can serve as an important tool for the survival of rhinoceros species, as it forms a protected environment and can lead to successful reintroductions into the wild. In the white rhinoceros, gestation length is estimated to be 16-18 months [Fouraker and Wagener, 1996; Patton et al., 1999] and the estrous cycle inferred from hormone analyses and behavior is approximately 32-38 days [Hindle et al., 1992; Radcliffe et al., 1997], although literature also reports a cycle length of 65-70 days due to a prolonged luteal phase [Hindle et al., 1992; Schwarzenberger et al., 1993; Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001]. Both cycle lengths can occur in one individual, and it is suspected that only the shorter cycles are fertile and can result in pregnancy

[Schwarzenberger et al., 1998; Brown et al., 2001]. At this stage it is still unclear which underlying mechanisms contribute directly to the occurrence of the longer cycles and whether there is a correlation with the high incidence of pregnancy failures in captivity. To ensure long-term health and self-sustaining populations, reproduction must be improved and inbreeding in small captive populations avoided [Wildt et al., 1997]. Techniques for manipulating ovarian cycles to allow artificial insemination (AI) combined with semen cryopreservation tools may provide a functional vehicle necessary to improve breeding success and avoid inbreeding depression within the captive population [Hermes et al., 2004; Hildebrandt et al., 2007].

The horse (*Equus caballus*), another Perissodactyla species, is often used as a model for reproduction in rhinoceros species [Roth, 2001]. Respective ultrasound studies in the black, white and Sumatran rhinoceros have shown that early pregnancy and embryo development are very similar in these species to that in the horse [Radcliffe et al., 1997; Radcliffe et al., 2001; Roth et al., 2001; Roth et al., 2004]. In the cycling mare, the administration of a synthetic progestagen, which suppresses folliculogenesis, results in a more precisely timed estrus [Squires, 2008; Samper, 2009]. The same response to a synthetic progestagen treatment, in this case altrenogest, has been documented in the white rhinoceros [Hermes et al., 2006; Hermes et al., 2012]. When close timing of the moment of ovulation is required, an ovulation-inducing hormonal agent can be added to the protocol. The three most common agents that are being used for induction of ovulation in mares are GnRH analogues

(agonists), human chorionic gonadotropin and recombinant equine luteinizing hormone (eLH) [Blanchard et al., 1998; Vanderwall et al., 2007; Squires, 2008]. One advantage of using a GnRH agonist to induce ovulation is that efficacy does not decrease after repeated use of GnRH agonists, as its small molecular weight reduces the chance that antibodies are being developed against it [Mumford et al., 1995; Bradecamp, 2007]. Also, protocols using GnRH agonists result in a more accurately controlled ovulation, as the induced ovulation occurs over a relatively small period, permitting a single fixed-time insemination [Gordon, 2004].

Several studies have involved attempts to induce estrus in the southern white rhinoceros [Godfrey et al., 1990; Walzer and Schwarzenberger, 1995; Hermes et al., 2012] and in the critically endangered Sumatran rhinoceros (*Dicerorhinus sumatrensis*), an induced ovulator with only nine individuals remaining in captivity [T. Roth et al., 2001; T. L. Roth, 2006; Stoops et al., 2011]. In a recent, unpublished study by M.W. Schook et al. (personal communication) a synthetic progestagen was used to induce estrus in the Indian rhinoceros (*Rhinoceros unicornis*). In another study, estrus was induced successfully in the Sumatran rhinoceros [T. L. Roth et al., 2004]. In 2012, Hermes et al., published a paper describing the use of a synthetic progestagen to induce estrus in the white rhinoceros, followed by different combinations of ovulation inducing agents [Hermes et al., 2012]. However, only small progress has been made and ovulation using those protocols has been inconsistent from female to female.

5.2.1. Aims and strategy

The present study was designed to validate a technique for inducing ovulation in the captive southern white rhinoceros by investigating the effects of synthetic progestagen and slow-release GnRH analogue on the initiation of estrus and ovulation respectively. The long-term goal was to develop a functional and reliable protocol for zoos and breeding facilities that can fit into assisted reproduction management plans. The benefits of implementing assisted reproduction techniques in rhinoceros conservation management are profound, in terms of improving timed breeding pregnancy outcomes, enabling the exchange of genetic material, overcoming physical and behavioral disabilities in individuals, embryo transfer and sex-ratio adjustments, and the encouragement of gamete- and embryo databases [AZA, 2014]. A second objective was to demonstrate usefulness of fecal progestagen metabolite (fPM) monitoring for tracking estrous cycle manipulations, making it possible to examine treatment outcomes non-invasively and eventually to realize the use of these tools in individuals in the wild.

5.3. Material and methods

5.3.1. Animals

Three mature female white rhinoceroses located at two facilities in the United States were used in estrus and ovulation induction trials (Table 5-1). Females A and B are mother (born December 1992, filial F1) and daughter (born November 2000, filial F2) and were housed together at Birmingham Zoo, Alabama. During the trial they were successfully introduced in the same enclosure to

a new breeding male. Female C, located at Jacksonville Zoo, Florida (born July 1994, filial F1), was housed together with a breeding male and had been monitored weekly for fecal endocrine hormones for a period of 11 months prior to the trial, using a polyclonal anti-progesterone raised in New Zealand White Rabbits against 11α-hemisuccinate progesterone, which has shown to reflect luteal activity reliably in monitored captive white rhinoceroses [Metrione L.M. et al., personal communication]. Females A and C were both multiparous proven breeders with no history of reproductive abnormalities. Female B had not produced offspring in the past but was considered sexually mature based on her age. Female A had been successfully trained for rectal ultrasound examination. Keeper staff scored observations of physical signs of estrus and/or socio-sexual behavior such as whistling, increased urination, mounting and mating [Owen-Smith, 1975]. Behaviors were recorded both in terms of frequency and duration of occurrence.

5.3.2. Steroid hormone extraction and enzyme immunoassay (EIA)

Individual fresh fecal samples were collected weekly in a period of 90 days prior to the onset of the trial for endocrine monitoring of estrous cycles. Samples were collected off the ground and were stored at -20 degrees Celsius until analysis. Crushed feces (0.48-0.52 gram) was added to 12×55mm extraction tubes (Fisher Scientific, Pittsburg, PA, USA) and extracted by centrifuging for 10 minutes at 3100rpm with added 0.5 mL deionized water and 4.5mL anhydrous ethanol [Metrione et al., 2008]. After centrifugation, an aliquot of extract supernatant was diluted 1:150 (females 1 and 2) or 1:500 (female 3) in EIA

Table 5-1. Background information of the white rhinoceros individuals used for this study.

Study animal	Date of Birth	Filial generation	Parity	Location
Female A	Dec 1992	F1	Multiparous	Birmingham Zoo
Female B	Nov 2000	F2	Nulliparous	Birmingham Zoo
Female C	Jul 1994	F1	Multiparous	Jacksonville Zoo

buffer for assaying and stored at -20°C [Metrione et al., 2008] (Appendix 4). The EIA of fecal steroid extracts was performed using the same polyclonal anti-progesterone (R4859), raised in New Zealand White Rabbits against 11a-hemisuccinate progesterone with working dilution 1:200,000 (Appendix 5), as was used to monitor one of the study animals during the 11 months prior to the trial. The progesterone antibody cross-reacts 40% with 11α-hydroxyprogesterone, 12.19% with 5α-pregnene-3, 20-dione, and < 0.05% with other steroids (Appendix 6). Fecal PM concentrations were expressed as mass units hormone per gram feces.

5.3.3. Hormonal treatment

To induce estrus 0.022mg/kg (female A and B) or 0.044mg/kg (female C) oral progestagen, altrenogest (Regu-mate®, Intervet America Inc., Millsboro, DE), which has shown to be effective in the Indian rhinoceros [Schook, M.W. et al, personal communication] was given daily for 21 days on the food, followed by a single intramuscular injection of slow-release GnRH analogue, deslorelin acetate (Sucromate™ Equine, Thorn

BioScience LLC, Louisville, KY) on day 30.5 [Hermes et al., 2012], with day 1 being the start date of the 21-day altrenogest treatment. The period between stopping oral progestagen treatment and administering GnRH analogue is 9.5 days, which corresponds with the documented follicular phase length in the white rhinoceros of 9.7-10.5 days of which the last two days are presumed to be the fertile window [Radcliffe et al., 1997; Patton et al., 1999; Hermes et al., 2012]. Onset of treatment in females A and B was chosen randomly. In female C treatment was started in the mid-luteal phase of an ovarian cycle, determined by ultrasound examination.

5.3.4. Data analysis

Hormone profiles were evaluated on indication of ovarian cyclicity and elevations in fPM concentration were considered to indicate a luteal phase if at least two values exceeded baseline level for at least 2 consecutive weeks (Ahlers et al., 2012; Brown et al., 2001). Baseline levels of fPM (ng/g) are presented as a horizontal line and were calculated using Brown's iterative method, excluding any values exceeding the mean plus 2 SD and recalculating averages [Brown et al., 1994; Brown et al., 2001]. Physical and/or behavioral signs of estrus were aligned with episodes of elevated fPM concentrations. Data are presented as mean ± SEM.

5.4. Results

During and after treatment, no behavioral or physical signs of estrus were seen in all three females. Females A and B were successfully introduced to a new male on day 109 of sample collection, two days after altrenogest treatment. Female C was observed breeding with the resident bull on day 5 of sample collection, 17 days before the start of treatment with altrenogest.

Baseline fPM concentrations for females A, B and C were calculated individually and were 3854.70, 3397.27 and 6142.69 ng/g feces, respectively (Fig. 5-1A,B,C). In all three females, elevations in fPM concentrations above baseline levels were present, indicating luteal phases with fPM concentrations reaching maximum values of 5466.86, 5523.34 and 9494.81 ng/g feces, respectively.

Fecal PM concentrations in both females A and B increased above baseline levels from day 94 of sample collection, during altrenogest treatment, and remained elevated for 21 and 20 days respectively, indicating luteal acitivity (Fig. 5-1A,B). In female C, fPM concentrations reached luteal values for 21 days from day 45 to day 66 of sample collection (Fig. 5-1C) and for 25 days from day 66.

Two days after GnRH analogue administration, fPM concentrations exceeded baseline levels in female B and remained elevated for 26 days until the last day of sample collection, indicating the start of a luteal phase (Fig. 5-1B). No luteal activity could be detected after administration of GnRH analogue in females A and

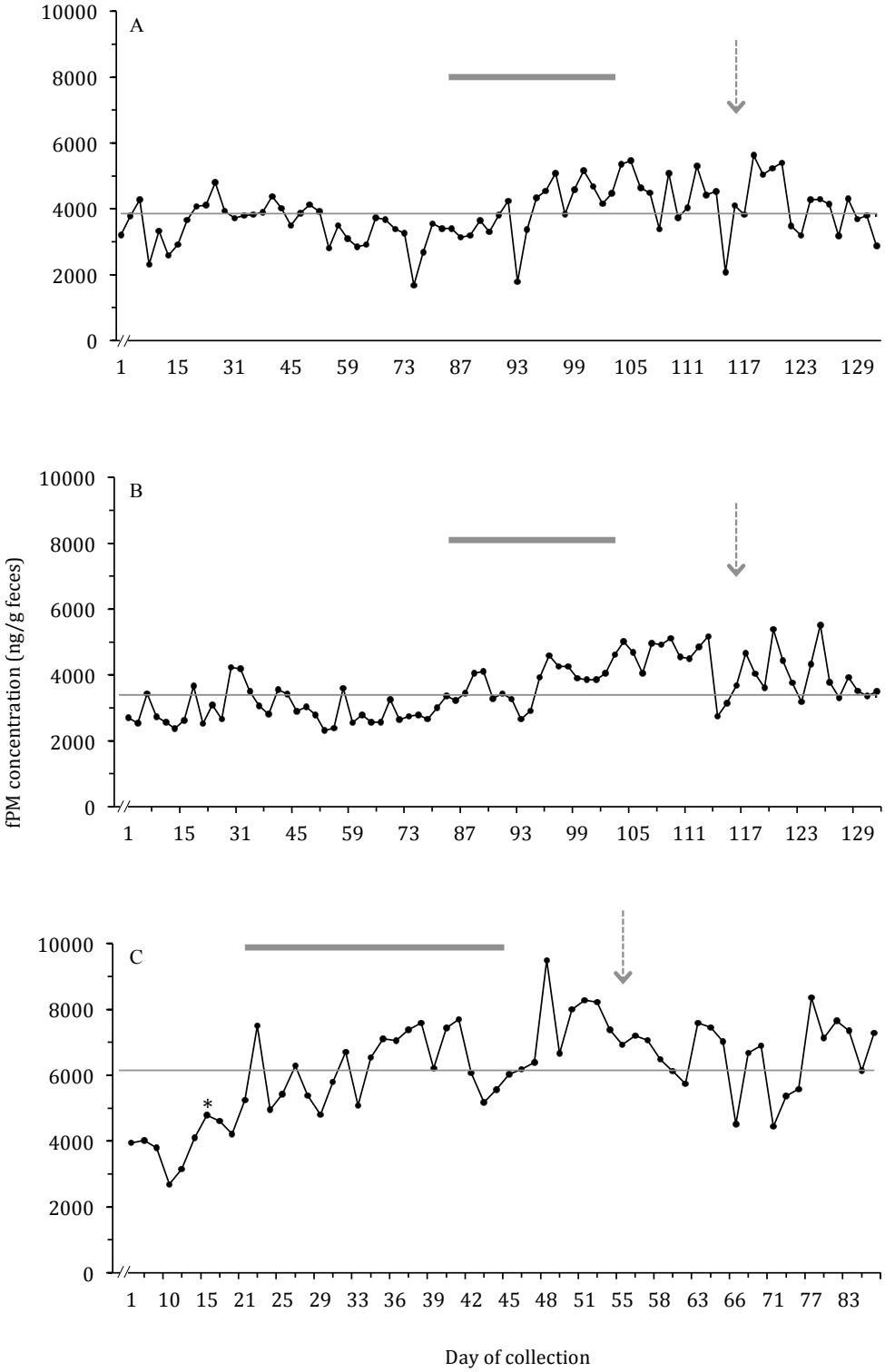

Figure 5-1. Longitudinal profiles of immunoreactive 11α-hemisuccinate progesterone concentrations (●) for 3 adult female white rhinoceroses before, during and after ovulation induction treatment. Females A and B are mother and daughter and were housed together at Birmingham Zoo. Female C, housed at Jacksonville Zoo, was bred on day 15 of sample collection (*). Altrenogest (Regu-mate®) was given for 21 days (grey bar). On day 30.5 an intramusculair injection of GnRH analogue deslorelin acetate (Sucromate™ Equine) was administered, after which ovulation can be expected (arrow). Individual fPM baseline levels are indicated (horizontal line).

C, which was administered on day 116 and day 55 of sample collection, respectively (Fig. 5-1A,C).

5.5. Discussion

The aim of this study was to validate a method to induce estrus and ovulation in the southern white rhinoceros and to demonstrate usefulness of fPM monitoring for tracking estrous cycle manipulations. Longitudinal profiles of the fecal concentrations of progesterone metabolites were characterized and fPM concentrations increased to luteal values in all three females, indicating the occurrence of an estrous cycle (n=4). After GnRH administration, only one female in this study showed a period of luteal activity in her hormone profile. It is therefore not possible to conclude that this was the result of our treatment protocol. Additionally, the increase started on the same day GnRH was administered. Brown et al. (2001) found that it takes 48 hours for an “event” to show up in the feces and therefore it is possible that the luteal phase detected here is not as a result of our treatment protocol, but more likely the result of a natural ovulation.

The estrous cycle of the mare is 22-23 days, with a follicular phase of 4-7 days [Blanchard et al., 1998]. After treatment with a synthetic progestagen for 8-10 days, mares generally begin to exhibit estrous behavior in 3 to 4 days and ovulation can be expected 7-8 days after treatment [Squires, 2008]. A GnRH analogue is given within 40 hours prior to anticipated ovulation to induce follicular maturation and ovulation [McKinnon and Voss, 1993; Ferris et al., 2012]. In this study, the GnRH

analogue deslorelin acetate was given 9.5 days after ending the synthetic progestagen treatment [Hermes et al., 2012]. It is still unclear around which day after treatment ovulation takes place in the white rhinoceros, for this reason the mare is being used as a model to determine the ideal moment of GnRH treatment. It is possible however, that the ideal moment to administer GnRH after an altrenogest treatment in the white rhinoceros needs to be adjusted, and future studies should focus on narrowing down the right time of administration.

In general, in case of an ovulation, progesterone will be produced by the corpus luteum and a rise in fPM hormone measured in the feces can be expected [Schwarzenberger et al., 1998]. Not every estrous cycle results in an ovulation. While the production of estrogens by the dominant follicle results in signs of estrus and a rise in LH, ovulation failure is a common problem seen in mares, with a higher incidence in older mares [McCue et al., 2007], and has been documented in the white rhinoceros [Hermes et al., 2012]. When a dominant follicle does not ovulate, it can turn into a luteal structure that produces progesterone [McCue et al., 2007]. This event can mimic the occurrence of an ovulation followed by a luteal phase, which must be considered when interpreting fecal hormone concentrations.

During treatment, fPM concentrations increased to luteal levels for 20-21 days in two females that were housed together. In the white rhinoceros, the follicular phase of the estrous cycle is approximately 9.7-10.5 days [Radcliffe et al., 1997; Patton et al., 1999]. This length applies for both “short” and “long” cycles, since the difference in length between these two

documented cycle types in white rhinoceros has been ascribed to variation in luteal phase length [Patton et al., 1999]. The luteal phase in the event of a “short cycle” of ~30-35 days could therefore be estimated to be 19.5-25.3 days. The observation in this study suggests a synchronized estrous cycle in these females because the elevation in both females started and ended within 24 hours of each another. It is not possible that this observed synchronization was the result of the estrus synchronization and ovulation induction protocol, and other causative factors could have initiated the synchronized onset of luteal activity in these females. One possible explanation for the increase in fPM concentrations during the treatment with altrenogest would be that this exogenous progestagen is what was being detected by the assay. It has been previously reported, however, that the administration of a synthetic progestagen does not affect fecal progestagen EIA measurements in a white rhinoceros [Walzer and Schwarzenberger, 1995]. Another possibility is that both females came into estrus naturally. Although it cannot be ruled out that the females were synchronized by chance, it seems highly unlikely as both females were acyclic prior to observed luteal activity. A naturally synchronized estrus in more than one female white rhinoceros has not been previously documented [Patton et al., 1999; Kuneš and Bičík, 2002; Carlstead and Brown, 2005; Metrione, 2010].
One female was bred prior to treatment but within the period of sample collection. Based on this behavioral observation it can be concluded that this female went through an estrous cycle during this period. However, no elevation in fPM concentration to luteal values could be detected after this event until 30

days later. This strongly suggests that our test method did not pick up an existent luteal phase and reasons for this must be further explored.

During treatment, staff keepers did not record any behavioral or physical signs of estrus. However, it is common in the mare that estrus and ovulation occur in the absence of estrous behavior when the mare is already in estrus at the start of treatment [Samper, 2009]. This could explain why fPM concentrations increased to luteal levels in the absence of estrous behavior. Until now, most studies that focused on inducing ovulation in the white rhinoceros involved (a combination of) single case studies [Godfrey et al., 1990; Walzer and Schwarzenberger, 1995; Schwarzenberger et al., 1998; Hermes et al., 2006; Hildebrandt et al., 2007; Hermes et al., 2012]. Hermes et al. (2012) described case studies in Europe using a protocol of chlormadinone acetate, combined with either hCG or GnRH analogues to induce estrus [Hermes et al., 2012]. Effects were assessed by fecal hormone analyses and a single post treatment ultrasound examination in 29 individuals. In 10.3% of these individuals a corpus luteum was detected, followed by a 30-day luteal phase in 100% of the cases. Another 82.8% did show a pre-ovulatory follicle, however 29.1% showed no subsequent luteal activity, suggesting that ovulation did not necessarily occur in these cases. Of the females that did show a pre-ovulatory follicle and subsequent luteal activity, the majority showed 70-day cycles, which could potentially describe a persistent preovulatory follicle. More studies with repeated ultrasound examination post treatment should be followed-up to assess these proposed explanations.

A synchronization protocol combining synthetic progestagen with estradiol-17β (P&E) to enhance follicular regression should be further explored, which is currently the most effective therapy to reliably induce timed estrus in mares [Samper, 2009]. Furthermore, it is effective in mares to give a single treatment of a luteolytic agent, prostaglandin, on the last day of the P&E treatment to destroy any potentially functional luteal tissue [Vanderwall et al., 2007]. Important improvements that will be helpful in identifying factors affecting ovulation success and in determining the ideal timing of drug administration in future protocol design are the duration of sample collection post-treatment, the replication of sample analysis to avoid test errors and the use of ultrasonography to evaluate follicle size to predict the onset of estrus and ovulation. In the white rhinoceros, a preovulatory follicle will ovulate when the diameter is approximately 30mm [Radcliffe et al., 1997] and by monitoring its growth prior to ovulation, the moment of GnRH analogue administration can be more precisely timed, increasing the chance of correctly-timed ovulation.

In this study, the method used to measure fecal concentrations of progesterone metabolites might have affected the results in this study negatively. As a follow up control study, it is therefore planned to reanalyze the samples obtained in this study with an enzyme immunoassay that has been successfully used to monitor fecal progesterone metabolites in the white rhinoceros (Goot et al. 2013). Clearly, the improvement of a functional estrus induction protocol would contribute to the evolvement of advanced reproductive techniques in the white rhinoceros and could potentially play a big role in white

rhinoceros breeding management, both in captivity and in the wild.

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5.7. References

AZA, (2014). Rhino Advisory Group's 2014 Research Masterplan. American Zoo and Aquarium Association.

Bertschinger, H. J. (1994). Reproduction in black and white rhinos: a review, Proceedings of a symposium on rhinos as game ranch animals. Onderstepoort, Republic of South Africa, 9-10 September 1994, 96-99.

Blanchard, T. L., Varner, D.D. & Schumacher, J. (1998). *Manual of Equine Reproduction*. St. Louis, Mosby-Year Book, Inc.

Bradecamp E. A. (2007). Estrous synchronization. In: *Current Therapy in Equine reproduction* (Samper, J.C., Pycock, J.F., and McKinnon, A.O. eds.), 22-25, Saunders, St. Louis.

Brown, J. L., Wasser, S.K., Wildt, D.E., & Graham, L.H., 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biology of Reproduction 51, 776-786.

Brown, J. L., Bellem, A.C., Fouraker, M., Wildt, D.E. & Roth, T.L. (2001). Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring, Zoo Biol., 20(6), 463-486.

Carlstead, K. & Brown, J.L. (2005). Relationships between patterns of Fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros, Zoo Biol., 24(3), 215-232.

Ferris, R. A., Hatzel, J.N., Lindholm, A.R.G., Scofield, D.B. & McCue, P.M. (2012). Efficacy of Deslorelin Acetate (SucroMate) on Induction of Ovulation in American Quarter Horse Mares, Journal of Equine Veterinary Science, 32(5), 285-288.

Fouraker, M. and T. Wagener (1996), AZA Rhinoceros husbandry resource manual, 1-67.

Godfrey, R. W., Pope, C.E., Dresser, B.L., Bavister, B.D., Andrews J.C. & Olsen, J.H. (1990). An attempt to superovulate a southern white rhinoceros (*Ceratotherium simum simum*), Theriogenology, 33, 231.

Goot, A. C. van der, Dalerum, F., Ganswindt, A., Martin, G.B., Millar, R.P. & Paris, M.C.J. (2013). Faecal Progestagen Profiles in Wild Southern White Rhinoceros (*Ceratotherium simum simum*), Afr. Zool., 48(1), 143-151.

Gordon, I. (2004). *Reproductive technologies in farm animals*. CABi, 91-92.

Hermes, R., Hildebrandt, T.B. & Göritz, F. (2004). Reproductive problems directly attributable to long-term captivity–asymmetric reproductive aging, Anim. Reprod. Sci., 82, 49-60.

Hermes, R., Hildebrandt, T.B., Walzer, C., Göritz, F., Gray, C., Niemuller, C. & F. Schwarzenberger, F. (2012). Estrus induction in white rhinoceros (*Ceratotherium simum*), Theriogenology, 78(6), 1217-1223.

Hermes, R., Hildebrandt, T.B., Blottner, S., Walzer, C., Silinski, S., Patton, M.L., Wibbelt, G., Schwarzenberger, F. & Göritz, F. (2005). Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation, Theriogenology, 63(1), 219-238.

Hermes, R., Hildebrandt, T.B., Walzer, C., Göritz, C.F., Patton, M.L., Silinski, S., Anderson, M.J., Reid, C.E. Wibbelt, G., K. Tomasova, K. &Schwarzenberger, F. (2006). The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*), Theriogenology, 65(8), 1492-1515.

Hildebrandt, T. B., Hermes, R., Walzer, C., Sós, E., Molnar, V., Mezösi, L., Schnorrenberg, A., Silinski, S., Streich, J., Schwarzenberger, F. & Göritz, F. (2007). Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation, Theriogenology, 67(9), 1473-1484.

Hindle, J. E., Most, E. & Hodges, J.K. (1992). Measurement of urinary oestrogens and 20{alpha}-dihydroprogesterone during ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses, J. Reprod. Fertil., 94(1), 237-249.

Kuneš, M. & Bičík, V. (2002). Social and sexual behaviour in captive breeding groups of white rhinoceros, Biologica, 39, 40.

McCue, P. M., Samper, J., Pycock, J. & McKinnon, A. (2007). Ovulation failure. In: *Current Therapy in Equine Reproduction*, 83-85.

McKinnon, A. & Voss, J. (1993). *Equine Reproduction.* Philadelphia, Lea & Febiger.

Metrione, L. C., Norton, T.M., Beetem, D. & Penfold, L.M. (2008). Seasonal reproductive characteristics of female and male Jackson's hartebeest (Alcelaphus buselaphus jacksoni), Theriogenology, 70(6), 871-879.

Metrione, L. C., L. M. Penfold, L.M. & Waring, G.H. (2007). Social and spatial relationships in captive southern white rhinoceros (*Ceratotherium simum simum*), Zoo Biol., 26(6), 487-502.

Milliken, T., Emslie, R.H. & Talukdar, B. (2009). African and Asian rhinoceroses – status, conservation and trade. A report from the IUCN Species Survival Commission (IUNC/SSC) African and Asian Rhino Specialist Groups and TRAFFIC to the CITES Secretariat. CoP15 Doc. 45.1, 1-18, The Hague, November 2009, CITES Secretariat.

Mumford, E.L., Squires, E.L., Jochle, E., Harrison, L., Nett, T.M. & Trigg, T.E. (1995). Use of deslorelin short-term implants to induce ovulation in cycling mares during three consecutive estrous cycles. An. Repro. Sc., 39, 129-40.

Owen-Smith, R. N. (1992). *Megaherbivores: the influence of very large body size on ecology*. Cambridge University Press.

Owen-Smith, R. N. (1975). The Social Ethology of the White Rhinoceros, Ceratotherium simum (Burchell 1817*), Zeitschrift für Tierpsychologie, 38(4), 337-384.

Patton, M. L., Swaisgood R.R., Czekala, N.M., White, A.M., Fetter, G.A., Montagne, J.P., Rieches, R.G. & Lance, V.A. (1999). Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior, Zoo Biol., 18(2), 111-127.

Prothero, D. R. & Schoch, R.M. (1989). *The Evolution of perissodactyls*. Oxford University Press.

Radcliffe, R. W., Czekala, N.M. & Osofsky, S.A. (1997). Combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): Preliminary results, Zoo Biol., 16(5), 445-456.

Radcliffe, R. W., Eyres, A.I., Patton, M.L., Czekala, N.M. & Emslie, R.H. (2001). Ultrasonographic characterization of ovarian events and fetal gestational parameters in two southern black rhinoceros *(Diceros bicornis minor)* and correlation to fecal progesterone, Theriogenology, **55,** 1033–1049.

Reid, C. E., Versteege, L., Ludwig, A. & Hermes, R. (2012). Analysis of the European captive Southern white rhinoceros (*Ceratotherium simum simum*) population using the International Studbook for the African White Rhinoceros (2001-2004) and integrating reproductive health findings, International Zoo Yearbook.

Roth, T.L., O'Brien, J., McRae, M., Bellem, A., Romo, S., Kroll, J. & Brown, J. (2001). Ultrasound and endocrine evaluation of the ovarian cycle and early pregnancy in the Sumatran rhinoceros, *Dicerorhinus sumatrensis*, Reproduction, 121(1), 139-149.

Roth, T. L., Bateman, H.L., Kroll, J.L., Steinetz, B.G. & Reinhart, P.R. (2004). Endocrine and ultrasonographic characterization of a successful pregnancy in a Sumatran rhinoceros (*Dicerorhinus sumatrensis*) supplemented with a synthetic progestin, Zoo Biol., 23(3), 219-238.

Roth, T. L. (2006). A review of the reproductive physiology of rhinoceros species in captivity, International Zoo Yearbook, 40(1), 130-143.Samper, J. C. (2009). *Equine breeding management and artificial insemination.* Philadelphia, WB Saunders Company.

Schwarzenberger, F., Francke, R. & Goltenboth, R. (1993). Concentrations of faecal immunoreactive progestagen metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*), J. Reprod. Fertil., 98(1), 285-291.

Schwarzenberger, F., Walzer, C., Tomasova, K., Vahala, J., Meister, J., Goodrowe, K.L., Zima, J., Strauß, G. & Lynch, M. (1998). Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*), Animal Reproduction Science, 53(1-4), 173-190.

Squires, E. L. (2008). Hormonal Manipulation of the Mare: A Review, Journal of Equine Veterinary Science, 28(11), 627-634.

Stoops, M. A., Bateman, H.L., Campbell, M.K. & Roth, T.L. (2011). Attempted in Vitro Maturation and Fertilization of Postmortem Sumatran Rhinoceros (*Dicerorhinus sumatrensis*) Oocytes, Journal of Zoo and Wildlife Medicine, 42(4), 723-726.

Swaisgood, R. R., Dickman, D.M. & White, A.M. (2006). A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females, Biological Conservation, 129(4), 468-476.

Vanderwall, D. K., Newcombe, J.R., Samper, J.C., Pycock, J.F. & McKinnon, A.O. (2007). *Current Therapy in Equine Reproduction*. St. Louis, Saunders Elsevier.

Versteege, L. (2012). European Studbook White Rhino (*Ceratotherium simum simum*). White Rhinoceros European Endangered Species Program. Annual Report 2012.

Walzer, C. & Schwarzenberger, F. (1995). Estrous induction in a white rhinoceroes (*Ceratotherium simum simum*) and concomitant EIA fecal progestagen analysis. Proceedings of the American Association of Zoo Veterinarians.

Wildt, D. E., Rall, W.F., Critser, J.K., Monfort, S.L. & Seal, U.S. (1997). Genome Resource Banks, Bioscience, 47(10, AIBS: The First 50 Years), 689-698.

Chapter 6

General discussion

The main objective of this thesis was to identify key characteristics in the reproductive endocrinology of the free-living female white rhinoceros and to address critical physiological similarities and differences between captive and wild individuals. A non-invasive method was developed that allowed for successful monitoring of fecal progestagen metabolites on a regular basis in the feces of wild female white rhinoceroses, reflecting important reproductive events such as pregnancy and ovarian cyclicity. The results supported the hypotheses that were being tested, namely that estrous cycles can be described in the wild white rhinoceros using non-invasive techniques and that, under normal circumstances, white rhinoceros females only show one length of estrous cycle. Additionally, an emphasis was placed in this thesis on the extreme value of the development of assisted reproduction technology in the future management of the white rhinoceros.
In the last few decades, much progress has been made in the development of non-invasive monitoring techniques to establish reproductive hormone patterns of a large variety of wildlife species both in captivity and in the wild [Pukazhenthi and Wildt, 2004; Schwarzenberger, 2007; Schwarzenberger and Brown, 2013]. This tool creates a great potential towards a better understanding of reproductive mechanisms in species in which invasive methods such as blood collection are not practical on a

regular basis and in which breeding seems problematic [Schwarzenberger, 2007; Schwarzenberger and Brown, 2013]. While numerous studies have focused on characterizing the reproductive cycle in most rhinoceros species in a captive environment, the collection of fecal samples for female reproductive hormone analysis in the wild has only been reported in the black rhinoceros (*Diceros bicomis minor*) [J. N. Garnier et al., 1998; J. Garnier et al., 2002]. This thesis provides the first description of reproductive hormone analysis for wild female white rhinoceroses ranging freely in their natural habitat. Results in this study have proven it to be feasible to collect samples on a regular basis from non-collared white rhinoceros females in a medium sized reserve. The main challenge was the remoteness and elusiveness of the focal animals and sample collection success rates did vary between individuals. Because a white rhinoceros normally defecates 3-4 times per day, of which one occurs during night time [Owen-Smith, 1992], one recommendation to increase sample collection success rates would be the use of advanced tracking technologies, as this could increase the chance of finding a focal animal before it has defecated early in the morning or late afternoon.

The enzyme immunoassay that was used to measure fPM concentrations in the collected fecal samples has been used successfully in a number of species, including the African elephant [Ganswindt et al., 2003; Szdzuy et al., 2006; Benavides Valades et al., 2012; Ahlers et al., 2012]. The endocrine data presented in this thesis confirms that the technique used can successfully detect pregnancy and ovarian cyclicity in the white

rhinoceros, and can also be useful for surveillance strategies or for the detection of fetal death during pregnancy.

An average ovarian cycle length of 30.6 days and a gestation length of 502 ± 3 days were found for the white rhinoceros in this study, which both resemble the published ovarian cycle and gestation length found in captivity [Schwarzenberger et al., 1998; Patton et al., 1999]. No “long cylces” could be detected suggesting that the “long cycles” described in captive white rhinoceros females have a pathological origin. However, more wild individuals would need to be monitored over a longer period of time to further test this hypothesis.

In this study, fPM concentrations increased above luteal phase concentrations in the 3rd month of gestation. Similar findings have been reported previously, mentioning high fPM concentrations in pregnant captive females during the 4th and 5th month of pregnancy [Schwarzenberger et al., 1998], while one study found a clear increase 2 months after ovulation [Hermes et al., 2012]. It can therefore be concluded that the non-invasive method developed in this study can be used for diagnosis of pregnancy in wild white rhinoceroses without the need to immobilize and/or relocate the animal. Currently, pregnancy diagnosis in wild rhinoceroses is oftentimes achieved through opportunistically collected blood samples during essential immobilisation for other purposes. A non-invasive alternative as presented in this study can therefore provide a useful tool for *in situ* management plans.

An interesting finding was that periods of acyclicity were detected and appeared related to the presence of a new adult bull, suggesting that white rhinoceros females in the wild might

need external stimuli to ovulate. While the white rhinoceros is considered to be a spontaneous ovulator, it was discovered in 2001 that the Sumatran rhinoceros is an induced-ovulator and that irregular cycling as well as luteinization of follicles is a common observation in this species in the absence of mating [Roth et al., 2001]. We therefore hypothesize that the white rhinoceros might be an induced ovulator, which could also partially explain the long cycles observed in captivity. Further investigation combining ultrasonography and fPM monitoring in captive and wild individuals is however needed to evaluate this theory.

The measurement of estrogens is being used in other Perissodactyla to reliably reflect pregnancy [Bamberg et al., 1991; Schwarzenberger et al., 1991; Chapeau et al., 1993], and the detection of urinary estrogens has been proven successful in the female white rhinoceroses [Hindle and Hodges, 1990; Hindle et al., 1992]. Although the measurement of fecal estrogen metabolite (fEM) concentrations in the white rhinoceros could provide a practical tool to reliably identify estrus and pregnancy, some studies reported a lack of increase in fEM concentrations during gestation in the white rhinoceros [Berkeley et al., 1997; Brown et al., 2001]. Unless an appropriate assay can be validated [Hodges et al., 2010], it is likely that the measurement of fEM concentrations might not be effective in the white rhinoceros [Berkeley et al., 1997; Brown et al., 2001].

This thesis also provides a next step towards the development of a functional protocol for synchronization of estrus and ovulation in the white rhinoceros, which has been proven to be

challenging partly because of the irregular cycling many females display [Godfrey et al., 1990; Hildebrandt et al., 2007; Hermes et al., 2007; Hermes et al., 2012]. In this study, the synthetic oral progesterone altrenogest (Regu-mate®, Intervet America Inc., Millsboro, DE) was tested in combination with the GnRH analogue deslorelin acetate (Sucromate™ Equine, Thorn BioScience LLC, Louisville, KY) for its efficiency to induce ovulation in two multiparous females and one nulliparous adult female. Luteal activity was detected during the trial in all study animals and two females displayed a spontaneous synchronized luteal phase. Natural ovarian synchrony in females that are housed together has not yet been reported in the white rhinoceros. However, the observations of luteal activity in this study did not seem to be related to the treatment protocol as fPM concentrations mostly started rising above baseline levels before and during treatment, prior to the administration of GnRH analogue after which follicular maturation and ovulation should be induced. The treatment protocol tested in this study did therefore not seem effective and improvement of the protocol is essential, with a view to offering timed natural breeding and assisted reproduction in the white rhinoceros.

Over the last few years there has been a massive increase in poaching of the African rhinoceros species and there is no single solution to end this highly organized crime [Emslie and Knight 2014]. Field security in the wild remains one of the most important and efficient, yet also one of the most expensive, ways to prevent poaching [Knight, 2011]. Other strategies such as educating the public, improving law enforcement and keeping the remaining populations healthy and breeding remain just as

important for the survival of this species [Milledge, 2007; Milliken et al., 2009; Knight, 2011]. Currently the option to legalize the horn trade is being explored [Biggs et al., 2013]. It is, however, very difficult to predict how the rhino horn market will respond to such drastic measures. Unfortunately, a great amount of corruption is involved in the trafficking of rhino horn and one can strongly question whether any change in the legal system will decrease the illegal killing of rhinoceros through the backdoor for profit. However, legalizing horn trade will most certainly generate an extra income for financially struggling rhino owners, who will then be able to sell their stockpiles and add value to the rhinoceroses they own [Biggs et al., 2013]. Once a rhinoceros becomes a valuable umbrella species, which currently is not the case, the owner is likely to put more effort and money into protecting its animals, which could aid in the conservation of the remaining populations of rhinoceros [Martin, 2012; Biggs et al., 2013].

With the wild populations being under severe threat, rhinoceros populations kept in captivity serve as a valuable genetic reservoir for future reintroductions into the wild. It is therefore critical that these populations breed successfully [Swaisgood et al., 2006]. This thesis presented a new approach toward understanding and solving the breeding problem seen in the white rhinoceros in captivity and has revealed major aspects of the reproductive physiology in the species. The findings afford further understanding of the low reproductive success observed in captivity, of which the underlying mechanisms and environmental root causes are yet unclear and not fully understood, by developing a more solid foundation of the normal

reproductive biology of the species. Results from this thesis have provided an insight into several aspects of the reproductive physiology of the white rhinoceros and have created an opportunity to integrate reproductive endocrine data from *in situ* white rhinoceros individuals in the assessment of non-reproductive females in captivity. The methods and techniques presented in this thesis also provide possible avenues for future research focusing on environmental factors regulating reproduction in white rhinoceros populations *in situ*, with the ultimate goal of improving breeding management strategies, both in captivity and in the wild. These strategies remain critical for the conservation and, ultimately, for the survival of this endangered species.

6.1. References

Ahlers, M. J., A. Ganswindt, S. Münscher, and H. J. Bertschinger (2012), Fecal 20-oxo-pregnane concentrations in free-ranging African elephants (*Loxodonta africana*) treated with porcine zona pellucida vaccine, Theriogenology, 78(1), 77-85.

Bamberg, E., E. Möstl, M. Patzl, and G. J. King (1991), Pregnancy diagnosis by enzyme immunoassay of estrogens in feces from nondomestic species, Journal of Zoo and Wildlife Medicine, 73-77.

Benavides Valades, G., A. Ganswindt, H. Annandale, M. L. Schulman, and H. J. Bertschinger (2012), Non-invasive assessment of the reproductive cycle in free-ranging female African elephants (*Loxodonta africana*) treated with a gonadotropin-releasing hormone (GnRH) vaccine for inducing anoestrus, Reproductive Biology and Endocrinology, 10, 63.

Berkeley, E. V., J. F. Kirkpatrick, N. E. Schaffer, W. M. Bryant, and W. R. Threlfall (1997), Serum and fecal steroid analysis of ovulation, pregnancy, and parturition in the black rhinoceros (*Diceros bicornis*), Zoo Biol., 16(2), 121-132.

Biggs, D., F. Courchamp, R. Martin, and H. P. Possingham (2013). Legal trade of Africa's rhino horns. *Science*, *339*(6123), 1038-1039.

Brown, J. L., A. C. Bellem, M. Fouraker, D. E. Wildt, and T. L. Roth (2001), Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring, *Zoo Biol., 20*(6), 463-486.

Chapeau, C., G. J. King, and E. Bamberg (1993), Fecal estrogens in one primate and several ungulate species during various reproductive stages, Anim. Reprod. Sci., 34(2), 167-175.

Emslie, R. H. and M. H. Knight (2014), Update on African rhino status and trends: from IUCN SSC African Rhino Specialist Group (AfRSG), in Report to CITES Standing Committee 65th meeting, 1-6.

Ganswindt, A., R. Palme, M. Heistermann, S. Borragan, and J. K. Hodges (2003), Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth, Gen. Comp. Endocrinol., 134(2), 156-166.

Garnier, J. N., D. I. Green, A. R. Pickard, H. J. Shaw, and W. V. Holt (1998), Non-invasive diagnosis of pregnancy in wild black rhinoceros (*Diceros bicornis minor*) by faecal steroid analysis, Reprod. Fertil. Dev., 10(6), 451-458.

Garnier, J., W. Holt, and P. Watson (2002), Non-invasive assessment of oestrous cycles and evaluation of reproductive seasonality in the female wild black rhinoceros (*Diceros bicornis minor*), Reproduction, 123(6), 877-889.

Godfrey, R. W., C. E. Pope, B. L. Dresser, B. D. Bavister, J. C. Andrews, and J. H. Olsen (1990), An attempt to superovulate a

southern white rhinoceros (*Ceratotherium simum simum*), Theriogenology, 33, 231.

Hermes, R., F. Göritz, W. Streich, and T. Hildebrandt (2007), Assisted Reproduction in Female Rhinoceros and Elephants – Current Status and Future Perspective, Reproduction in Domestic Animals, 42(s2), 33-44.

Hermes, R., T. B. Hildebrandt, C. Walzer, F. Göritz, C. Gray, C. Niemuller, and F. Schwarzenberger (2012), Estrus induction in white rhinoceros (*Ceratotherium simum*), Theriogenology, 78(6), 1217-1223.

Hildebrandt, T. B., R. Hermes, C. Walzer, E. Sós, V. Molnar, L. Mezösi, A. Schnorrenberg, S. Silinski, J. Streich, F. Schwarzenberger, and F. Göritz (2007), Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation, Theriogenology, 67(9), 1473-1484.

Hindle, J. E. and J. K. Hodges (1990), Metabolism of oestradiol-17{beta} and progesterone in the white rhinoceros (*Ceratotherium simum simum*), J. Reprod. Fertil., 90(2), 571-580.

Hindle, J. E., E. Most, and J. K. Hodges (1992), Measurement of urinary oestrogens and 20{alpha}-dihydroprogesterone during ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses, *J.Reprod.Fertil., 94*(1), 237-249.

Hodges, K., J. Brown, and M. Heistermann (2010), Endocrine monitoring of reproduction and stress, Wild mammals in captivity: principles and techniques for zoo management, 447-468.

Knight, M. (2011). African Rhino Specialist Group report. *Pachyderm* 49*:* 6-15.

Martin, R. (2012). A legal trade in rhino horn: Hobson's choice*,* Rhino Survival Trust, Johannesburg, South Africa.

Milledge, S. A. (2007). Illegal killing of African rhinos and horn trade, 2000-2005: the era of resurgent markets and emerging organized crime. *Pachyderm*, (43), 96-107.

Milliken, T., R. H. Emslie, and B. Talukdar (2009). African and Asian rhinoceroses – status, conservation and trade. A report from the IUCN Species Survival Commission (IUNC/SSC) African and Asian Rhino Specialist Groups and TRAFFIC to the CITES Secretariat. CoP15 Doc. 45.1, 1-18, The Hague, November 2009, CITES Secretariat.

Owen-Smith, R. N. (1992), *Megaherbivores: the influence of very large body size on ecology*, reprint, illustrated ed., 388.

Patton, M. L., R. R. Swaisgood, N. M. Czekala, A. M. White, G. A. Fetter, J. P. Montagne, R. G. Rieches, and V. A. Lance (1999), Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior, *Zoo Biol., 18*(2), 111-127.

Pukazhenthi, B. S. and D. E. Wildt (2004), Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod. Fertil. Dev., 16(2), 33-46.

Roth, T., J. O'Brien, M. McRae, A. Bellem, S. Romo, J. Kroll, and J. Brown (2001), Ultrasound and endocrine evaluation of the ovarian cycle and early pregnancy in the Sumatran rhinoceros, *Dicerorhinus sumatrensis*, Reproduction, 121(1), 139-149.

Schwarzenberger, F., E. Mostl, E. Bamberg, J. Pammer, and O. Schmehlik (1991), Concentrations of progestagens and oestrogens in the faeces of pregnant Lipizzan, trotter and thoroughbred mares, J. Reprod. Fertil. Suppl., 44, 489-499.

Schwarzenberger, F., C. Walzer, K. Tomasova, J. Vahala, J. Meister, K. L. Goodrowe, J. Zima, G. Strauß, and M. Lynch (1998), Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white

rhinoceros (*Ceratotherium simum*), Animal Reproduction Science, 53(1-4), 173-190.

Schwarzenberger, F. (2007), Non-invasive endocrine monitoring using fecal steroid analysis: opportunities and challenges, Revista Brasileira de Zootecnia, 36, 87-88.

Schwarzenberger, F. and J. Brown (2013), Hormone monitoring: an important tool for the breeding management of wildlife species, Wiener Tierärztliche Monatsschrift, 100, 209-225.

Swaisgood, R. R., D. M. Dickman, and A. M. White (2006), A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females, *Biological Conservation, 129*(4), 468-476.

Szdzuy, K., M. Dehnhard, G. Strauss, K. Eulenberger, and H. Hofer (2006), Behavioural and endocrinological parameters of female African and Asian elephants *Loxodonta africana* and *Elephas maximus* in the peripartal period, International Zoo Yearbook, 40(1), 41-50.

Chapter 7

Appendices

Appendix 1 – Steroid extraction protocol

(Chapters 3 and 4)

A. Receiving Samples:

1) Please check for the following:

   - Faecal samples have to be frozen (if samples are received dry, continue with step 3)

   - Alternatively evaporated faecal extracts might be received (see separate protocol for the reconstitution of those extracts)

   - Discuss further proceeding for other sample matrices with Professor A. Ganswindt

   - Sample containers have to be labelled properly (Sample ID, Animal ID, Date, and any further relevant information)

   - Sample list has to accompany the samples / be provided (preferably in excel format)

2) Store faecal samples at -20°C until freeze-drying and label storage bags/boxes clearly

3) Open a project folder and info sheet for the new project, enter all available data and information (see project information template).

B. Freeze-Drying:

1) Ensure that samples are thoroughly frozen prior to lyophilisation
2) Check freeze dryer for working condition (oil, seals, general appearance; clean trays if necessary)
3) Start pump and freezer
4) Open containers, remove caps/stoppers, place containers on the trays
5) Close the freeze-dryer (dry samples according to manufacturer's instructions)
6) Clean and dry caps/stoppers
7) Check for pressure and temperature regularly, enter into log-file provided
8) Remove samples from freeze-dryer when complete dryness has taken place depending on number and volume of samples (usually between 48-72 hours) and close containers.
9) Store dry faecal samples at room temperature.

C. Pulverisation:

You will need: 80% ethanol in a spray bottle, paper to place underneath, sieves, tweezers, waste bags, paper towel roll, and the dry faecal samples. Please wear laboratory coat, gloves, and surgical mask

1) Clean all surfaces with 80% ethanol prior to pulverisation and between the samples, and wipe dry
2) Clean sieve and tweezers prior to pulverisation and between the samples with 80% ethanol, and wipe dry
3) Work on a piece of paper, empty entire contents of the container into the sieve placed on the paper

4) Work faecal powder through the sieve with tweezers by moving sample around in the sieve
5) Fill faecal powder back into the container, remove coarse material that remained in the sieve into the prepared waste bag, and discard paper
6) Clean and dry the surface and tools before starting with the next sample
7) Take a new piece of paper, and continue with pulverising the next sample
8) Continue for all samples (make notes of, e.g. odd looking samples, mislabelling etc.)
9) Store faecal powder in labelled box at room temperature
10) Have the waste incinerated.

D. Weighing:

You will need scales (at least accurate to the mg), spatula, 80% ethanol, tissue paper, rack with properly labelled tubes (5 ml) and lids for all samples, sample list containing an empty column for writing down the sample weight, pen

1) Clean surfaces, scales, spatula prior to weighing and clean spatula between samples
2) Put empty labelled tube (cap removed) on the scale, set weight to zero
3) Weigh 0.050 g to 0.060 g of faecal powder of the respective sample
4) Write down the exact weight of faecal powder in the sample list
5) Close tube and put the tube back into the sample rack

6) Clean spatula with 80% ethanol, wipe dry and continue with the next tube and sample
7) Have the waste incinerated
8) Enter sample weights into prepared excel sample list.

E. Steroid Extraction:

You will need a multi-tube vortex, a centrifuge with a respective rotor fitting for the used tubes, lab timer, multi-stepper pipette, 50 ml syringe, 80% ethanol (freshly prepared), properly labelled micro centrifuge tubes (1.5 ml) for all extracts

1) Remove caps from the tubes and put them in order to ensure individual caps will be replaced on the correct tubes
2) Add 3 ml 80% ethanol (unless stated otherwise) per sample, preferably using a multi-stepper pipette
3) Vortex on a multi-tube vortex for 15 min; please ensure that all of the faecal powder is in suspension before starting
4) Centrifuge the tubes for 10 min at 1500 x G
5) Double-check matching labels on tubes and micro-centrifuge-tubes, and decant supernatant into the respective micro-centrifuge-tubes, taking care not to stir up the pellet
6) Discard the remaining excess supernatant unless discussed otherwise
7) Let the pellets dry in the tubes, and discard afterwards unless discussed otherwise (e.g. if needed for determination of organic weight; see separate protocol)

8) Store steroid extracts in labelled box at -20°C until used for EIA analysis.

Endocrine Research Laboratory
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2013

Appendix 2 – Cross-reactivity data for the 5α-pregnan-3β-ol-20-one enzyme immuno assay

Steroid	% Cross-reactivity
5α-pregnan-3β-ol-20-one	650
5α-pregnan-3β-ol-20-one	100
4-pregnen-3,20-dione (progesterone)	72
5α-pregnan-3,20-dione	22
5β-pregnan-3β,20α-diol	<0.1
4-pregnen-20α-ol-3-one	<0.1
5b-pregnan-3β-ol-20-one	<0.1
5α-pregnan-20α-ol-3-one	<0.1
5α-pregnan-3β,20α-diol	<0.1
5α-pregnan-3β,20α-diol	<0.1

Source: Szdzuy, K., Dehnhard, M., Strauss, K., Eulenberger, K. & Hofer, H. 2006. Behavioural and endocrinological parameters of female African and Asian elephants. *International Zoo Yearbook* 40: 41–50.

Appendix 3 – Enzyme immuno assay protocol

(Chapters 3 and 4)

A. Preparation:

1) Prepare protocol sheet and accompanying assay sample list according to the templates
   - Adjust dilution factor of antibody and steroid label on the protocol sheet if necessary
   - Determine dilution factors on the assay sample list
   - Prepare respective excel file (results table) for subsequent calculations and data analysis
2) Check if all reagents, buffers, and solutions are fresh and available
3) Check if coated plates are available
4) Check if all necessary pipettes for the respective assay are available
5) Locate the faecal extracts needed for the assay
6) Check if the respective EIA protocol in the Gen5 software is available.

B. EIA day 1:

1) Defrost, sort and dilute the faecal extracts according to the assay sample list
2) Defrost respective standard, quality controls (QCs), antibody, steroid label, and coated microtiter plate
3) Prepare standard curve serial dilutions with assay buffer according to the protocol sheet
4) Add assay buffer to antibody and labelled steroid according to the protocol sheet

5) Wash the coated plate 4 times with 300 µl washing solution per well in the washer, pat dry Label plate on the side using permanent marker (assay type, project name, date)
6) Pipette 100 µl assay buffer into wells A1 + A2, pipette 50 µl assay buffer, standard, QCs and diluted extracts into the respective wells
7) Pipette 50 µl labelled steroid into every well using a multipette
8) Pipette 50 µl antibody into every well (**EXCEPT** into the BLANK A1 + A2) using a multipette
9) Cover the plate with cling wrap, mix contents carefully so that no loss of volume occurs, and place the plate into fridge over night.

C. EIA day 2:

1) Defrost streptavidin-POD aliquot (20 µl), add to 16 ml of cool assay buffer, rinse tube twice
2) Discard the contents of the plate into the BSA waste, wash the plate 4 times with 300 µl cool washing solution per well, pat dry
3) Pipette 150 µl streptavidin-POD solution into every well using a multipette
4) Incubate the plate in the fridge on the plate shaker for 45 min
5) Discard the contents of the plate into the BSA waste, wash the plate 4 times with 300 µl cool washing solution per well, pat dry
6) Mix 250 µl TMB with 17 ml cool substrate using solution directly before use

7) Pipette 150 µl substrate solution into every well using a multipette
8) Incubate plate in the fridge on plate shaker until OD of the zero wells is about 1.0, normally 30-60 minutes (check for colour change after 5 min for the first time)
9) Start computer and printer, open respective protocol in the Gen5 software, start the reader only briefly before use
10) Stop the enzyme reaction by carefully adding 50 µl H_2SO_4 (2M) per well using a multipette
11) In Gen5, enter the plate information into the experiment window, carefully place the plate onto the reader carrier and read the plate
12) Take the plate out of the reader and let it dry at RT on lab bench
13) Print out the results, export results into excel file, transfer the excel file data into pre-prepared file on your computer and analyse the results (see below).

D) Analysis of results:

1) Determine linear range for the plate on the print-out
2) Determine duplicate outliers and linear-range-outliers for re-measurement
3) Transfer results to be used to the prepared Excel results table and calculate concentrations
4) Transfer results of the QCs together with the added information into the quality control list

5) Mark the samples for re-measurement in the results table and determine new dilution factors for the linear-range-outliers.

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Appendix 4 – Wet weight shaking extraction protocol (Chapter 5)

Preparing Samples for Extraction:

1. Sort fecals by date. Record dates & comments on extraction sheet. If an individual has previous extractions, use same sheet to continue with current samples.

2. Crush & mix fecal pellets using rubber mallet into powdered consistency until a fine, granular grit forms. This works best if fecals are frozen, especially the ungulate pellets. Felids work best slightly thawed.

3. Label 16x100mm extraction tubes with assigned extraction numbers.

4. Weigh out 0.500gram (0.480-0.520g) well-mixed sample into tubes. Use as clean a sample as possible (actual fecal material vs. hair, straw, pine needles, dirt, etc.). Record actual weight. Record unusual consistency or debris as comments. Between samples, rinse weigh spoon with water & then isopropyl alcohol. Wipe with paper towel.

5. Cap vials. If necessary, weighed samples can be frozen until extraction. Samples should be kept frozen when possible.

6. Save a *small* amount of excess fecal material (enough to weigh out a couple more samples). *The larger bulk of it should be thrown out* to conserve space in freezers. Label bag as “Extracted” with date range and extraction numbers. Pack tightly & as compactly as possible. Return to freezer.

Extraction Process:

1. Label set of 12x55mm tubes for each dilution being saved (usually 3 sets- 500FL dried, 1:10, & working dilution, if known). Use computer-generated labels. Specify species/accession #/collection date/dilution/sample number. Ex.: Gerenuk #980032, 15 May 00 1:10 #16

2. Add 0.5mL RO water and 4.5mL anhydrous ethanol to each tube. Recap tubes immediately after adding alcohol.

3. Place the rack(s) of tubes in plastic bag. Caps must be on tight. Lock in place on Glas-Col Large Capacity Mixer. Use foam pads top & bottom to enable tight lockdown of samples. Shake 15 minutes with speed set at ~90.

5. Place tubes in centrifuge being careful to balance them. Centrifuge 10 minutes at ~3100 rpm.

6. Use one set of labeled 12 x 55mm tubes for dried extracts. Working quickly with ethanol extract, pipette 500 FL into 12 x 55mm tube & recap extraction tube. 12 x 55mm tubes remain uncapped for evaporation. Once dry, cap tubes & freeze.

7. For 1:10 dilutions: Use repeater pipette to add appropriate volume EIA buffer to each 12 x 55mm tube. Then, working quickly, add required volume ethanol extract to EIA buffer & cap tube. Example: for 1.0mL of a 1:10 dilution: use 900FL EIA buffer and 100FL extract.

8. Repeat procedure for other dilutions using appropriate volumes. For example:
   For 1:40 = use 975FL buffer & 25FL extract
   For 1:200 = use 950FL buffer & 50FL of 1:10 dilution

9. Store dilutions in cryo-boxes. Label boxes with appropriate color tape & species, accession #/house name & dilution. Add to an individual's currently existing boxes until filled. **Store in freezer.**

Washing Extraction Tubes

Only the large (16 x 100mm) plastic extraction tubes & caps are cleaned & reused.

Step 1. Superficially rinse items under faucet. For 16 X 100 mm extraction tubes, vortex to loosen fecal pellet & dump into “used alcohol” container for proper disposal & rinse. Using Terg-A-Zyme Powdered Detergent at ~ 2 ½ Tbsp./gallon of water, soak in warm, soapy water.

Step 2. Using scrubby pad, scrub extraction #s off tubes.

Step 3. Using brush, scrub items inside & out. Rinse thoroughly under faucet.

Step 4. Rinse once with RO water.

Step 5. Set items to dry on clean towel. Prop upright, if possible.

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Appendix 5 – Enzyme immuno assay protocol

(Chapter 5)

1. Plate Coating	* Add 35.3 µL antibody stock (1:50, -20^{o}C) to 6 mL coating buffer for a working dilution of 1:8,500 * Add 50 µL/well using repeater pipette * Do not coat NSB wells * Tap plates gently to ensure that coating solution covers well bottom * Label & cover with plate sealer to avoid evaporation * Incubate overnight at 4^{o}C.
2. Plate Washing	* Wash the plate 4 times with wash solution * Blot on paper towel to remove excess wash solution * Proceed to next step quickly; do not allow plate to dry
3. Blocking	* Add 50 µL EIA buffer to all wells using repeater pipette * Cover with plate sealer; plate can sit for up to 3 hours
4. Standards	* Standard values are 1000, 500, 250, 125,

	62.5, 31.2, 15.6, 7.8, 3.9 & 0 vpg/well * Dilute standard stock (1000 pg/well or 20ng/mL) serially 2-fold using 200 µL stock & 200 µL EIA buffer (the 0 standard is buffer only-do not add standard)
5. Samples	* Dilute samples in EIA buffer to the appropriate dilution
6. HRP	* Working dilution is 1:200,000 * Add 4.5 µL stock (1:150, 4°C) to 6.0 mL EIA buffer
7. Plate loading	* Add 50 µL standard, control or sample per well in duplicate as quickly & accurately as possible, according to plate map * Immediately add 50 µL/well diluted HRP * Entire load time should be under 10 minutes * Cover with plate sealer & using plate shaker, shake (setting = 4.5) 2 hours at room temperature
8. Plate washing	* wash plate 4 times with wash solution & blot dry * plates are stable at this point & can be left until all plates are washed

9. Substrate	* prepare ABTS substrate immediately before use * combine 40 µL 0.5M H_2O_2, 125 µL 40 mM ABTS & 12.5 mL substrate buffer & mix well * add 100 µL substrate to all wells using repeater pipette * replace plate sealer & incubate at room temperature on plate shaker until optical density of zero standards reads about 1.000
10. Plate Reading	* read at 405nm (test filter 1, reference filter 4)

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Appendix 6 – Cross-reactivity data for the 11a-hemisuccinate progesterone enzyme immuno assay

Steroid	Cross-reactivity %
4-pregnen-3,20-dione (progesterone)	100.00
11α-OH-progesterone	40.00
5α-pregnan-3,20-dione	12.19
17α-OH-progesterone	0.38
20α-OH-progesterone	0.13
20β-OH-progesterone	0.13
Pregnanediol	<0.01
Pregnenolone	0.12
Estradiol 17β	<0.01
Estrone	<0.01
Testosterone	<0.01
Cortisol	<0.04

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