

Establishing indicators of the reproductive state in the captive Southern white rhinoceros (*Ceratotherium simum simum*) by combining behavioural, hormonal and bioacoustic measures

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Southern white rhinoceros (*Ceratotherium simum simum*) by
combining behavioural, hormonal and bioacoustic measures

THESIS

Submitted in partial fulfilment of the requirements for the degree of
Doctor of Natural Sciences

Doctor rerum naturalium

(Dr. rer. nat.)

awarded by the University of Veterinary Medicine Hannover

by

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Kharkiv, Ukraine

Hannover, Germany 2021

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Date of final exam: 14th April 2021

This research was funded by the *Serengeti-Park Stiftung* and the *Deutsche Forschungsgemeinschaft* (SCHE 1927/2-1)

*To Shaka –
thank you for reminding me of nature's power*

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Excerpts

Chapter 2 of this thesis has been previously published in a peer-reviewed journal:

Jenikejew J, Chaignon B, Linn S & Scheumann M (2020)

Proximity-based vocal networks reveal social relationships in the Southern white rhinoceros

Scientific Reports 10, 15104

<https://doi.org/10.1038/s41598-020-72052-0>

Chapter 3 of this thesis has been previously published in a peer-reviewed journal:

Jenikejew J, Wauters J, Dehnhard M & Scheumann M (2021)

The female effect - How female receptivity influences testosterone levels, behaviour and vocalization in male Southern white rhinoceroses

Conservation Physiology 9, coab026

<https://doi.org/10.1093/conphys/coab026>

Chapter 4 of this thesis is prepared to be submitted to a peer-reviewed journal:

Jenikejew J, Wauters J, Dehnhard M & Scheumann M

Linking socio-sexual and vocal behaviour with faecal progesterone and oestrogen metabolite levels in Southern white rhinoceros females

Conferences

Parts of the thesis have been presented at the following scientific conferences:

Graduate meeting in Animal Behaviour, Hannover, Germany, July 12th-13th, 2018.

Jenikejew J, Chaignon B, Linn S & Scheumann M

“Project description: Interview with a rhino – Does vocalisation encode social relationships and hormone states in the Southern white rhinoceros (*Ceratotherium simum simum*)?”

ASAB Winter Meeting, London, UK, December 6th -7th, 2018.

Jenikejew J, Chaignon B, Linn S & Scheumann M

“Interview with a rhino – How vocalisations reflect social interactions in the captive Southern white rhinoceros (*Ceratotherium simum simum*)”

14th Annual Meeting of the Ethologische Gesellschaft e.V., Hannover, Germany, February 6th – 8th, 2019.

Jenikejew J, Chaignon B, Linn S & Scheumann M

“Interview with a rhino – How vocalisations reflect social interactions in the captive Southern white rhinoceros (*Ceratotherium simum simum*)”

27th International Bioacoustics Congress, Brighton, UK, August 31st – September 5th, 2019.

Jenikejew J, Chaignon B, Linn S & Scheumann M

“Interview with a rhino – functionality and directionality of communication calls in the captive Southern white rhinoceros”

15th Annual Meeting of the Ethologische Gesellschaft e.V., Tübingen, Germany, February 18th – 21st, 2020.

Jenikejew J, Chaignon B, Linn S & Scheumann, M

“Interview with a rhino – establishing communication structure and call functionality in a semi-social species”

List of abbreviations

AS	association strength
CV	coefficient of variability
DC	direct contact
EAZA	European Association of Zoos and Aquaria
EEP	EAZA <i>ex situ</i> programme
EIA	enzyme immunoassay
fEM	faecal oestrogen metabolites
fPM	faecal progesterone metabolites
fTM	faecal testosterone metabolites
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
h	hour(s)
HPLC	high-performance liquid chromatography
HPA	hypothalamic-pituitary-adrenal axis
HQC	high quality control
ION	ratio between node in- and outdegree in a network
IOW	ratio between weight in- and outdegree in a network
IUCN	International Union for Conservation of Nature
LH	luteinising hormone
LM	linear model
LME	linear mixed effect model
LQC	low quality control
SD	standard deviation
SM	sexually mature
SNM	sexually non-mature
SSC	Species Survival Commission
SWR	Southern white rhinoceros (<i>Ceratotherium simum simum</i>)
T	testosterone
WR	White rhinoceros (<i>Ceratotherium simum</i>)
yrs	years

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Summary

Julia Jenikejew (2021)

Establishing indicators of the reproductive state in the captive Southern white rhinoceros (*Ceratotherium simum simum*) by combining behavioural, hormonal and bioacoustic measures

The pressure on the survival of many animal species has increased enormously, particularly over the last century, due to anthropogenic effects and advancing climate change. This has called for urgent concepts designed to promote the preservation of biodiversity and conservation strategies. These concepts can be divided into two categories: measures that are implemented in the natural habitats (*in situ*) and those taking place in captivity (*ex situ*). Due to the fact that the conditions for successful *in situ* measures are becoming more difficult for logistical, political and climatic reasons, *ex situ* strategies are gaining in importance, especially for endangered or rare animal species.

One of the most important elements of *ex situ* conservation is captive breeding, which requires an elaborate and reliable reproduction monitoring to be successful. This includes both physiological and behavioural aspects, from which concrete measures can be derived for everyday handling of the respective animals. This is particularly relevant for those species known to have an impaired reproductive rate in captivity, such as the white rhinoceros (*Ceratotherium simum*).

In this species, classified as “near threatened” by the IUCN, previous studies particularly described endocrinological abnormalities in the oestrous cycle and pathologies of the female genital tract, leading to an asymmetrical reproductive age in captivity. In addition, poor sperm quality has been specified on the part of the males. Unfavourable group constellations, and thus social stress, are also believed to affect the animals’ fertility. To date, however, there has been a lack of studies that examined these aspects simultaneously thereby enabling a direct linkage between them. The aim of the present work is to close this gap, by complementing systematic behavioural observations and acoustic analyses with endocrinological studies in captive Southern white rhinoceroses (*Ceratotherium simum simum*).

Firstly, the vocal networks outlined in *Chapter 2* illustrate sex-specific differences in call rates for the contact call Pant and the aggressive calls Hiss and Grunt. Adult males acted defensively towards the females while using Pant calls for advertising, whereas adult females generally rejected the males and kept them at a distance with aggressive calls. The results not

only highlight the importance and function of vocal communication for the white rhinoceroses, but also show that the social dynamics within the groups correspond to those in the wild.

Chapter 3 examines behaviour and vocalisation as well as the concentrations of faecal testosterone metabolites (fTM) of adult males as a function of the receptivity of the females. The findings revealed that males who were kept in direct contact with cyclic females had significantly higher fTM concentrations than those who were either alone or without cyclic females. However, a comparison between the receptive and non-receptive periods of the females showed that the fTM concentrations of the males did not change during the cycle. In contrast, interaction and call rates increased significantly during the receptive period: males spent significantly more time in close proximity to the females, showed more socio-positive behaviour towards them and uttered Hiss calls, but specifically Pant calls almost exclusively during the fertile days of the females. Hence, the results confirm that female receptivity has a general effect on the androgen concentrations of the males, and they simultaneously reveal clear indicators in both the behaviour and vocalisation of the males during this period.

Finally, *Chapter 4* investigates the changes in female behaviour and vocalisation during their receptivity. These were examined for a potential coincidence with the time of their oestrus, which was determined by analysing faecal sex hormone metabolite concentrations. The hormone profiles of the progesterone metabolites (fPM) proved to be a reliable measure for the identification of the follicle and luteal phases, while the hormone profiles of the oestrogen metabolites (fEM) could not provide any information about the cycle phases. In the adult females that signalled their receptivity by accepting the advertising and sexual behaviour of the males, significant changes in behaviour could be observed during this period. Compared to the non-receptive period, these females spent more time in close proximity to the male and demonstrated significantly more socio-positive behaviour – especially by lifting up their tails, and displayed more marking behaviour. In the majority of females, receptivity coincided with the lowest levels of the fPM values, corresponding to their hormonal oestrus.

In conclusion, this thesis presents clear sex-specific differences in the reproductive behaviour and vocalisation of the white rhinoceros. While the males express their interest in receptive females and their motivation to mate mainly through acoustic communication, females signal their receptivity mainly through olfactory communication. These behavioural patterns reflect the natural social organisation of the species in the wild and are crucial for intact reproduction. Hence, effective *ex situ* reproduction monitoring should provide opportunities for these sex-specific behavioural patterns to be expressed, and the occurrence thereof to be considered when making breeding-related decisions.

Zusammenfassung

Julia Jenikejew (2021)

Ermittlung von Indikatoren für den Reproduktionsstatus des südlichen Breitmaulnashorns (*Ceratotherium simum simum*) in Zoos unter Einbezug von Verhaltens-, Hormon- und Bioakustikuntersuchungen

Besonders seit dem letzten Jahrhundert ist der Druck auf den Fortbestand vieler Tierarten aufgrund anthropogener Einflüsse und des fortschreitenden Klimawandels enorm gestiegen und fordert immer dringender Konzepte zum Erhalt der Biodiversität und dem einhergehenden Natur- und Artenschutz. Diese Konzepte lassen sich in zwei Kategorien unterteilen – Maßnahmen, die in den ursprünglichen Lebensräumen der Flora und Fauna eingesetzt werden (*in situ*) und diejenigen, die außerhalb der natürlichen Habitate in Menschenhand stattfinden (*ex situ*). Während die Bedingungen für erfolgreiche *in situ* Maßnahmen aus logistischen, politischen sowie klimatischen Gründen immer schwieriger werden, gewinnen die *ex situ* Strategien insbesondere für stark gefährdete oder seltene Tierarten mehr und mehr an Bedeutung.

Einer der wichtigsten Teilaspekte des *ex situ* Artenschutzes ist dabei die Zucht in zoologischen Institutionen, bei der vor allen Dingen ein ausgearbeitetes und bewährtes Reproduktionsmonitoring erfolgsentscheidend ist. Dieses schließt sowohl physiologische als auch verhaltensbasierte Aspekte ein, aus denen sich konkrete Maßnahmen für den alltäglichen Umgang mit den jeweiligen Tieren ableiten lassen. Dies ist besonders für die Tierarten relevant, bei denen unzureichende Reproduktionserfolge in Menschenhand bekannt sind. Hierzu zählt auch das von IUCN als „potenziell gefährdet“ eingestufte Breitmaulnashorn (*Ceratotherium simum*).

Bisherige Studien zu dieser Spezies haben vor allem bei den Weibchen ein asymmetrisches Reproduktionsalter beschrieben, welches sich durch endokrinologische Abnormalitäten im Zyklus und pathologische Ausbildungen im weiblichen Geburtskanal äußert. Auf Seiten der Bullen wurde unzureichende Spermienqualität aufgezeigt. Auch ungünstige Gruppen-Konstellationen und damit einhergehender sozialer Stress stehen im Verdacht einen Einfluss auf die eingeschränkte Reproduktionsrate zu haben. Bis dato mangelt es jedoch an Studien, die die genannten Aspekte parallel zueinander untersuchen und somit einen unmittelbaren Bezug herstellen könnten.

Die vorliegende Arbeit zielt darauf ab, diese Lücke zu schließen, indem systematische Verhaltensbeobachtungen und Rufanalysen bei südlichen Breitmaulnashörnern (*Ceratotherium simum simum*) in Zoos mit endokrinologischen Untersuchungen kombiniert werden.

Die in *Kapitel 2* generierten vokalen Netzwerke zeigten geschlechtsspezifische Unterschiede in den Rufraten für den Kontaktruf Pant und die aggressiven Rufe Hiss und Grunt. Während die adulten Bullen in den Gruppen eine defensive Rolle gegenüber den Weibchen einnahmen und den Pant-Ruf als Werberuf nutzten, waren die Weibchen den Bullen gegenüber die meiste Zeit ablehnend eingestellt und hielten sie mit aggressiven Rufen auf Abstand. Die Ergebnisse stellten dabei nicht nur die Relevanz und Funktion der vokalen Kommunikation für die Breitmaulnashörner heraus, sondern zeigten auch, dass die soziale Dynamik innerhalb der Gruppen derjenigen in der Wildnis entspricht.

In *Kapitel 3* wurden schließlich sowohl die Verhaltens- und Rufraten als auch die Konzentrationen der Testosteronmetaboliten in Kotproben (fTM) der adulten Bullen in Abhängigkeit der Rezeptivität der Weibchen untersucht. Dabei stellte sich heraus, dass Bullen, die im direkten Kontakt zu zyklischen Weibchen gehalten wurden, signifikant höhere fTM-Konzentrationen aufwiesen, als diejenigen die entweder allein oder ohne zyklische Weibchen waren. Ein Vergleich zwischen der rezeptiven Phase und den nicht-rezeptiven Phasen der Weibchen zeigte jedoch, dass sich die fTM-Konzentrationen der Bullen während des Zyklus nicht veränderten. Die Verhaltens- und Rufraten wiesen hingegen einen deutlichen Anstieg während der rezeptiven Phase auf: Die Bullen verbrachten signifikant mehr Zeit in der unmittelbaren Umgebung der Weibchen, zeigten ihnen gegenüber vermehrt sozio-positive Verhaltensweisen und äußerten den Hiss-Ruf, insbesondere aber den Pant-Ruf fast ausschließlich während der fruchtbaren Tage der Weibchen. Somit bestätigten die Ergebnisse einen allgemeinen Effekt der weiblichen Rezeptivität auf die Androgen-Konzentrationen der Bullen und offenbarten gleichzeitig deutliche Indikatoren sowohl im Verhalten als auch in der Vokalisation der Bullen für diese Phase.

Um das Bild des Reproduktionsmonitorings bei Breitmaulnashörnern zu vervollständigen, hat sich *Kapitel 4* abschließend den Veränderungen im Verhalten und in der Vokalisation der Weibchen während ihrer Rezeptivität gewidmet und diese auf die potenzielle Übereinstimmung mit dem Zeitpunkt ihres Östrus untersucht, der mit Hilfe der Analyse von Sexualhormonmetaboliten in Kotproben festgestellt wurde. Dabei erwiesen sich die Hormonprofile der Progesteronmetaboliten (fPM) als zuverlässiges Maß für die Identifikation der Follikel- und Lutealphasen, während die Hormonprofile der Östrogenmetaboliten (fEM) keinen Aufschluss über die Zyklusphasen liefern konnten. Bei den adulten Weibchen, die ihre

Rezeptivität durch die Akzeptanz des Werbe- und Sexualverhaltens seitens der Bullen signalisierten, konnten während dieser Phase signifikante Verhaltensänderungen beobachtet werden. Im Vergleich zu nicht-rezeptiven Phasen verbrachten die Weibchen mehr Zeit in unmittelbarer Nähe zum Bullen und demonstrierten sowohl deutlich mehr sozio-positive Verhaltensweisen, insbesondere durch das Abspreizen des Schwanzes, als auch mehr Markierverhalten. Bei der Mehrheit der Weibchen fiel diese Rezeptivitätsphase mit dem Tiefststand der fPM-Werten, entsprechend mit ihrem hormonellen Östrus zusammen.

Zusammenfassend stellt die Arbeit deutliche Geschlechtsunterschiede sowohl im Verhalten als auch der Vokalisation der Breitmaulnashörner im Reproduktionskontext fest. Während die Bullen ihr Interesse an rezeptiven Weibchen und ihre Fortpflanzungsmotivation vornehmlich durch akustische Kommunikation bekunden, signalisieren die Weibchen ihre Fortpflanzungsbereitschaft hauptsächlich durch die olfaktorische Kommunikation. Beide Schwerpunkte spiegeln die natürliche soziale Organisation der Spezies in der Wildnis wider und sind entscheidend für eine intakte Fortpflanzung. Für ein bestmögliches *ex situ* Reproduktionsmonitoring sollten daher alle geschlechtsspezifischen Verhaltensmuster sowohl durch entsprechendes Tiermanagement ermöglicht als auch bei Zucht-bezogenen Entscheidungen berücksichtigt werden.

1

General Introduction

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Chapter 1

General Introduction

1.1 | Modern zoos and their role in conservation

Last centuries' intensified anthropogenic activities – such as habitat destruction, overuse of natural resources, environmental pollution and poaching – led to extremely accelerated extinction rates of animal and plant species all over the world (ANDERMANN *et al.* 2020, BARNOSKY *et al.* 2011, DE VOS *et al.* 2015). In fact, the International Union for Conservation of Nature (IUCN) reports that almost 30% of all assessed species are currently threatened with extinction (IUCN 2021). The resulting loss in biodiversity has been aggravated by the processes of climate change and hence, has come to the fore of environmental and conservation research aiming to recover and sustain a thriving biodiversity (COMIZZOLI & HOLT 2019, MARTON-LEFÈVRE 2010). In this process, two strategies focusing on the maintenance of genetic diversity and population sustainability of endangered species in their natural habitat (*in situ*) and in captivity (*ex situ*) emerged (BRAVERMAN 2014, COMIZZOLI & HOLT 2019).

By definition, the *in situ* approach implies the restoration and maintenance of natural biotopes and ecosystems (PRITCHARD *et al.* 2012). These processes are often laborious and costly - and more often than not - limited by the prevalent political and logistic conditions as well as by human-wildlife conflicts (CONWAY 2011). Hence, in many cases the natural habitat is not available in time or long enough, to accommodate the flora and fauna in question (CONWAY 2011, PRITCHARD *et al.* 2012). With these constraints in view, preservation of viable populations in captivity and thus *ex situ* conservation is inevitable. Therefore, integrating both approaches – instead of implementing them separately – has recently been the main goal of the „*One Plan Approach*“, which has been proposed by the Species Survival Commission (SSC) of the IUCN, implying the aggregation of resources and data from both conservation communities, in order to create a common and comprehensive plan on conservation strategies and actions for all populations of a species (BYERS *et al.* 2013, SCHWARTZ *et al.* 2017, TRAYLOR-HOLZER *et al.* 2019). Respective guidelines have been published by IUCN SSC for conservationists, to evaluate the extent to which *ex situ* management could contribute to the overall conservation strategy for a specific taxon (IUCN/SSC 2014).

Ever since the involvement of zoological parks and aquariums (hereafter *zoos*) as *ex situ* conservation institutions, they have established a number of multidisciplinary programmes including the exchange with wildlife populations and the founding and support of *in situ* projects on the one hand and captive breeding on the other (BRAVERMAN 2014, SCHWARTZ *et al.* 2017). Thereby, captive breeding serves not only as a critical genetic and demographic

reservoir for reintroduction and population strengthening of endangered species, but also as one of the most essential connections between reproductive science and its practical implementation in the wild (OWEN *et al.* 2004). Over the past decades, numerous examples in various taxa – ranging from primates (KLEIMAN *et al.* 1986) through carnivores (HEDRICK & FREDRICKSON 2008, MILLER *et al.* 1994) and ungulates (KOŁODZIEJ-SOBOCIŃSKA *et al.* 2018, SPALTON *et al.* 1999) to birds (DOERR *et al.* 2017, LESOBRE *et al.* 2010) and reptiles (GIBBS *et al.* 2014, GRANT & HUDSON 2015) – have demonstrated the success of this conservation strategy, exemplifying the re-establishment of captive-born endangered animals into their native habitats (BECK *et al.* 1994, reviewed by CONWAY 2011, JULE *et al.* 2008).

Withal, one of the main tools that is used for the scientific management of captive breeding are international studbooks, which contain the lineage and demographic information on each captive individual of a specific taxon that is included in the programme (BARONGI *et al.* 2015). In addition, studbooks include records on rearing and information on particularities in behaviour or physiology that might be relevant for breeding. These records, being constantly updated and shared between various holders and studbook keepers, enable a global exchange on the management and monitoring of threatened and rare animal species (ZOOLOGICAL SOCIETY OF LONDON 2019). At the European level, *ex situ* programmes (EEP) have been established, which specifically coordinate the breeding of threatened species among the European Association of Zoos and Aquaria (EAZA)-facilities in order to maintain healthy, self-sustaining populations (EAZA 2021). One of the decisive elements for the success of EEPs are elaborate monitoring concepts for the species in question.

1.2 | Monitoring strategies in zoos

In order to ensure successful and sustainable breeding in captivity, many fundamental decisions and procedures concerning the animal in question – such as housing conditions, diet, group composition, enrichment measures and medical care – need to be thoroughly monitored (KLEIMAN *et al.* 2010). Thereby, monitoring describes a systematic collection and analysis of the animals' behavioural and physiological data in different contexts, providing information on relevant aspects, such as general well-being, activity profiles, stress level as well as their reproductive state (WHITHAM & WIELEBNOWSKI 2013). A vast number of studies has been investigating this kind of data in a variety of different species in captivity, attempting to draw management implementations that would enhance welfare, diversity of species-typical behavioural repertoire and ultimately the reproduction rate in the long-term.

For instance, studies on giant pandas have demonstrated that environmental enrichment, promoting different kinds of behavioural opportunities, leads to the reduction of stereotypic behaviour and increase in general activity (SWAISGOOD *et al.* 2001). In addition, enclosure designs with a higher complexity resulted in reduced aggression and increased contact between the breeding pair (reviewed by KLEIMAN 1994). Similarly, studies on tree-shrews (MARTIN 1968) and elephant-shrews (RATHBUN *et al.* 1981) have proven that adjusting the enclosures according to the animals' innate social organisation reduces agonistic behaviour and improves reproduction. In black rhinoceroses, a cross-institutional analysis showed that group composition, as well as size and design of the enclosures and the degree of public access are evident predictors of reproductive success (CARLSTEAD *et al.* 1999).

Nowadays, different strategies of animal monitoring are applied, often in combination with one another, in order to examine a wide range of indices and hence obtain results that are as conclusive as possible (HILL & BROOM 2009). Thereby, the most fundamental and evident method of behavioural monitoring is commonly complemented and put into context with endocrine monitoring (SHEPHERDSON *et al.* 2004, WHITHAM & WIELEBNOWSKI 2013). For some decades now, bioacoustic monitoring has evolved as an independent method as well (TEIXEIRA *et al.* 2019). All three approaches are carefully examined in the following.

1.2.1 | Behavioural monitoring

Behavioural monitoring incorporates the systematic observation and evaluation of all behavioural patterns of a group of animals or of an individual within a group in different contexts (HILL & BROOM 2009). In this process, a complete repertoire of all species-specific behaviours, also known as an ethogram (SCHLEIDT *et al.* 1984), is compiled and classified according to specific functional systems such as e.g., *social behaviour*, *foraging behaviour*, *marking behaviour* or *comfort behaviour* (LEHNER 1998). Usually, the functional systems are divided into further subcategories, as they are highly dependent on various modifiers, such as social environment and situational context. For instance, a distinction can be made between *agonistic* and *affiliative behaviour*, subsequently between *social exploration* and *sexual behaviour*, and finally the specific interaction partners that are involved (WATTERS *et al.* 2009).

Behavioural parameters are usually indicated as presence/absence, frequency or duration within a specific time period. Depending on the question and required information, different observational sampling methods can be chosen, e.g., *ad libitum sampling* (opportunistic sampling with no constraints), *focal animal sampling* (restriction of sampling to one individual/pair/group during specific period), *instantaneous/scan sampling* (sampling of an

individual/group at sequential, predetermined points in time) or *one-zero sampling* (sampling occurrence or non-occurrence of selected behaviour during sequential, predetermined points in time) (ALTMANN 1974, LEHNER 1992). In the recent past, the original approach of paper-and-pencil data collection has been substituted by video recording and subsequent analysis, using behavioural coding software, such as *EthoVision* (SPINK *et al.* 2001), *BORIS* (FRIARD & GAMBA 2016) and *Observer XT* (VAN DAM *et al.* 2013).

Behavioural monitoring provides various types of useful information for animal management in captivity. For instance, the prevalence as well as the regularity of certain behavioural patterns in captivity can be used to draw comparisons to the respective behaviour occurring in the wild. In doing so, stereotypy as well as absent, excessive or atypical responses to social or physical stimuli can call attention to potentially abnormal behaviour (HILL & BROOM 2009, HOSEY *et al.* 2013, MALLAPUR 2005). Based on these observations, species-specific indicators of stress response or discomfort could be deduced (OWEN *et al.* 2004). Conversely, behavioural patterns such as playing can serve as indicators for positive affective states (reviewed by YEATES & MAIN 2008). In addition, intra-specific affiliative behaviour such as grooming or close body contact can also be used to draw relevant conclusions: on species level, the respective behaviour can provide information on the mating system, while on group level it might give insights into the social relationships between the individuals (YOUNG 1998).

With regards to successful captive breeding the findings from behavioural observations are of major importance, as they can be used for the identification of both male and female willingness to mate, as shown in a variety of different mammalian species, such as southern hairy-nosed wombats (SWINBOURNE *et al.* 2018), koalas (JOHNSTON *et al.* 2000), zebu cattle (LLEWELYN *et al.* 1987), blackbucks (ARCHUNAN & RAJAGOPAL 2013), jaguars (STEHLIK 1971) and Asiatic lions (UMAPATHY *et al.* 2007). Thereby, typical female-specific behaviour that indicates their oestrus phase usually comprises increased overall activity, reduced food intake, altered body posture and generally accepting the sexual interactions initiated by the males (MEINECKE *et al.* 2010, SILVA *et al.* 2017). Other visible signs can be vaginal mucus discharge or vulvar swellings and discolouring (SILVA *et al.* 2017). However, in some species females have a rather silent or discrete oestrus, impeding the identification of their cycle phase based on external signs (SILVA *et al.* 2017).

Therefore, behavioural observation, although the most conventional method of animal monitoring, oftentimes needs to be complemented with other techniques to enable reliable conclusions. Besides, behavioural observation requires routine and experience not only regarding the tools of this method but also regarding the species and the animal in question

(WATTERS *et al.* 2009). Sound conclusions can therefore only be drawn from long-term and regular observations, where also inter-observer reliability must be ensured (WATTERS *et al.* 2009). Hence, while behavioural monitoring is certainly characterised by its intuitiveness and minimal technical requirements, it does involve expenditure of time and perseverance.

1.2.2 | Endocrine monitoring

The methodical monitoring of hormone concentrations of wild animals started more than half a century ago, with researchers linking behaviour and steroid plasma concentrations as well as analysing steroid levels in urinary samples, in order to access information on ovarian activity and pregnancy in captive non-human primates (GANSWINDT *et al.* 2012, reviewed by HIGHAM 2016). In the decades that followed, these methods have been established in a growing number of other species and by now include not only the tracking of reproductive states in males and females, but also glucocorticoids and their role as indicators of stress response (GANSWINDT *et al.* 2012).

Hormone concentrations can be measured in a variety of biological matrixes, such as blood, milk, saliva, faeces, urine, feathers and hair (HODGES *et al.* 2010, PALME 2012). The most accurate and immediate information on the endocrine status of an animal can be obtained from the hormone levels bound to transport proteins circulating from endocrine cells to the target cells in the blood stream (GRAHAM 2004, SCHWARZENBERGER & BROWN 2013). However, blood sample collection is an invasive process that is especially stressful for wild animals and there are only a few species that can be habituated to this routine (SCHWARZENBERGER & BROWN 2013). Hence, in order to avoid disturbing the animals and reduce stress as much as possible, non-invasive endocrinological approaches - based on urine and faecal samples - have been established in many different species (reviewed by BROWN 2018, HIGHAM 2016, SCHWARZENBERGER & BROWN 2013, SILVA *et al.* 2017).

Main steroid types in endocrine monitoring

Regarding endocrine monitoring in wild animals, four steroid classes have proven to be of particular relevance: Glucocorticoids (e.g., corticosterone and cortisol), androgens (e.g., testosterone), progestogens (e.g., progesterone) and oestrogens (e.g., oestradiol). Thereby, the precursor for the biosynthesis of all of these steroids is cholesterol that is converted to pregnenolone by the cholesterol side-chain cleavage enzyme in the first step of the steroidogenic pathway, thus providing a starting compound for the further conversion to sex hormones and adrenal gland hormones (HELDMAIER *et al.* 2013)

Testosterone concentrations usually provide information on the reproductive state of the male (e.g., PEI *et al.* 2009, THOMPSON *et al.* 2012), and its concurrence with mating season (e.g., PLACE & KENAGY 2000), whereas progesterone and oestrogen levels are commonly used for monitoring female reproductive activity (GANSWINDT *et al.* 2012). Furthermore, glucocorticoids have been used for the analysis of stress response ever since it was established that challenging and threatening situations result in an increased secretion of the steroid in the adrenal cortex (KAPPELER 2017, reviewed by WHITHAM & WIELEBNOWSKI 2013), potentially implying deleterious consequences for reproduction (reviewed by TILBROOK *et al.* 2000).

Monitoring of the reproductive state in males

In mammalian males, the reproductive state can be assessed via androgen measurements such as testosterone and its metabolites. The production and secretion of androgens is stimulated by Follicle-Stimulating Hormones (FSH) and Luteinising Hormones (LH) that belong to the family of gonadotropin hormones, which are synthesised and secreted by the gonadotropic cells of the anterior pituitary gland (HELDMAIER *et al.* 2013). In this process, LH activates the synthesis of testosterone in the Leydig cells, which are found adjacent to the seminiferous tubules in the testicles. In turn, testosterone supports the development of Sertoli cells that form the epithelium of the tubules and are therefore crucial for the process of spermatogenesis. At the same time, a negative feedback loop effect of testosterone and inhibin on the hypothalamus and pituitary keeps the FSH secretions at a constant level and as such, also the sperm-production (HELDMAIER *et al.* 2013).

The increase in testosterone concentrations during the process of sexual maturation has been described in numerous species and was attributed to the key role of testosterone as the main mediator for the development of male primary and secondary sexual characteristics (HELDMAIER *et al.* 2013). Moreover, positive correlations or a rise in testosterone levels were associated with mating efforts and intrasexual competition in males (e.g., CAVIGELLI & PEREIRA 2000, GLEASON *et al.* 2009). For some species, a direct link between changes in testosterone levels in males and the time point of oestrus of their mating partner could be established, emphasising the relevance of synchronised reproduction activity for seasonal breeders. For instance, in giant pandas (BONNEY *et al.* 1982, KLEIMAN 1983) the peak in male androgen levels coincides with the female's oestrus season. Similarly, in tammar wallabies (SCHNEIDER *et al.* 2010) and camels (YAGIL & ETZION 1980) the rise in male androgen levels concurs with the female's ovulation.

Monitoring of the reproductive state in females

Researchers determine the oestrous cycle and thus, the receptive phase of females based on the recurring endocrine changes reflected in the hormone profiles (HOBEIKA *et al.* 2020). In most mammalian females the oestrous phase is subdivided into follicular and the luteal phases (Figure 1.1). At the beginning of the follicular phase, neurons of the hypothalamus secrete the Gonadotropin-Releasing-Hormone (GnRH), which stimulates the pituitary to release FSH and LH in a pulsatile manner (HELDMAIER *et al.* 2013, MEINECKE *et al.* 2010). Both types of gonadotropin hormones act synergistically on the ovarian follicles. While FSH activates the maturation process of ovarian follicles by binding to the receptors of the granulosa cells that surround the oocyte thus stimulating the production of oestrogen and various growth factors, LH binds to the receptors of theca cells. This, in turn, stimulates the secretion of androgens, which are then transported to the granulosa cells to be aromatised into oestrogens. The growing follicles produce augmented levels of oestrogen, which subsequently stimulate the proliferation of the endometrium as well as granulosa cells and thus, FSH-binding receptors (MEINECKE *et al.* 2010). At the late stage of the follicular phase the *graafian* follicle reaches its fully matured state by dominating over the remaining follicle cohort that undergoes atresia (BROWN 2018, MEINECKE *et al.* 2010). The highly elevated levels of oestradiol produced by the dominant follicle result in a higher sensitivity of the pituitary for GnRH and consequently, in an LH surge that eventually induces ovulation – a process describing follicle disruption and the release of the oocyte into the fallopian tube (HELDMAIER *et al.* 2013). The beginning of the luteal phase is characterised by the luteinisation of the cells that ultimately form the *corpus luteum*, which produces increasing amounts of progesterone. The elevated progesterone levels facilitate the secretory phase of the endometrium, preparing it for the potential implantation of a blastocyst (HELDMAIER *et al.* 2013).

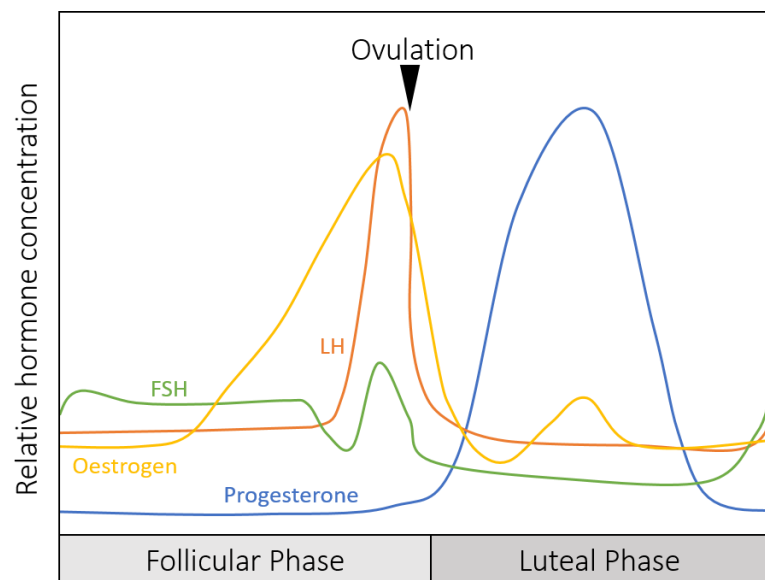


Figure 1.1: Schematic illustration of the relative hormone concentrations during an oestrous cycle in mammals. FSH: Follicle-stimulating hormone, LH: luteinising hormone.

In case of successful implantation, the lifespan of the *corpus luteum* is prolonged, as the elevated progesterone levels are vital for the embryo. The gestation period is characterised by the dominance of progesterone that is maintained until shortly before parturition, when progesterone levels decrease and oestrogen levels increase again (MEINECKE *et al.* 2010). If fertilisation or implantation did not occur, the endometrium is usually reabsorbed or shed (in humans, apes and some other species), initiating the beginning of a new cycle (HELDMAIER *et al.* 2013).

Long-term monitoring of the ovarian activity provides not only information on the time point of ovulation but also on potential abnormalities in the cycle, such as prolonged luteal phase, cycle irregularity or its complete absence (GRAHAM 2004). This information is of major importance for the breeding management of wild animals – especially of solitary species that require the separation of males and females until the onset of the oestrus phase (e.g., NORTH & HARDER 2008). In addition, endocrine monitoring can provide insights into potential pseudopregnancies (e.g., VAN DER WEYDE *et al.* 2015) or miscarriage (e.g., BEEHNER *et al.* 2006) as well as into the effects of age, or external factors on the female reproductive state (e.g., HUSSEIN *et al.* 2008, PACKER *et al.* 1998).

Limitations of endocrine monitoring

Non-invasive endocrine monitoring based on faecal or urine samples certainly presents the clear advantage of the non-necessity of animal handling over blood samples (GRAHAM 2004). However, at the same time the pooled endocrine values result in rather curbed hormone profiles that are less sensitive to more acute fluctuations, which might prove to be of particular importance (SCHWARZENBERGER 2007). In addition, while measuring hormones in blood or saliva is described as a straightforward procedure and a real-time reflection of the hormonal status with little to no time lag in the majority of cases (BROWN 2018), the measurement of hormones that pass through the route of excretion is not to be directly equated to the actual concentrations of the native hormone. This is due to the fact that mammals predominantly excrete hormone metabolites rather than the native kind (HIGHAM 2016, SCHWARZENBERGER 2007). Hence, it is necessary to determine the major metabolites found in faeces and urine first. This can be done either by injecting radioactively labelled hormones, thereby making all respective metabolites in the excreta distinguishable from metabolites of other origin, or by determination via chromatography or mass spectrometry, which identifies the components by their elution patterns or molecular weight, respectively (GRAHAM 2004, HIGHAM 2016, SCHWARZENBERGER 2007). Especially pioneering radiolabelling studies have proven that there

are usually no significant concentrations of native hormones found in faeces and urine and that the major metabolites, as well as their excretion pathways, vary to a great extent not only between species but also between hormone classes (reviewed by HIGHAM 2016). Therefore, each radio- or enzyme immunoassay applied to measure the concentrations of hormone metabolites needs to undergo separate testing and validation for each species. The implemented techniques are usually group-specific assays with high antibody cross reactions to various forms of a hormone class (GRAHAM 2004, HIGHAM 2016, HODGES *et al.* 2010, SCHWARZENBERGER 2007).

Overall, the establishment of a valid endocrine assay is an intricate but necessary process, which has still not been fully completed for many species (GANSWINDT *et al.* 2012). Furthermore, in addition to immediate storage at -20°C after sample collection, endocrine monitoring requires trained staff and the collaboration with specialised laboratory facilities. Therefore, endocrine monitoring provides clear physiological parameters that, however, imply elaborate technical and logistical requirements as well as complex data analysis.

1.2.3 | Bioacoustic monitoring

Bioacoustics describes the multidisciplinary field of analysing the production, dispersion and perception of acoustic signals on individual as well as species level (BRADBURY & VEHCAMP 1998, MCLOUGHLIN *et al.* 2019). Vocalisations can be recorded by microphones (on land) or hydrophones (in water) and analysed for their temporal (e.g., call rate, call duration, inter call interval ICI) as well as spectral (e.g., fundamental frequency F0, formant frequencies F1-N, amplitude) parameters, allowing a classification according to call type (BRADBURY & VEHCAMP 1998, WHITHAM & MILLER 2016). In addition, simultaneous behavioural observations enable the determination of context and function of call types and the identification of sender and receiver allows conclusions to be drawn regarding their social relationship (FICHEL & MANSER 2010, SNIJDERS & NAGUIB 2017). The multiparametric analysis of the vocalisations is usually conducted using a variety of sound analysis software, such as *Praat* (BOERSMA & WEENINK 2001), *BatSound* (PETTERSSON 2004) or *RavenPro* (BIOACOUSTICS 2019). Analysis is based on the analogue recordings of the vocalisations being scanned at a particular frequency, which are subsequently converted into digital recordings.

Acoustic signals, usually being wide-ranging and highly noticeable, gained more and more importance as a tool for wildlife conservation, as autonomous recording could provide information on occurrence, abundance as well as density – even for cryptic species that are otherwise difficult to detect or rare (MCLOUGHLIN *et al.* 2019, RISCH *et al.* 2013, TEIXEIRA *et*

al. 2019). However, in contrast to behavioural and endocrinological methods the implementation of bioacoustic techniques in animal monitoring is still less common, even though comprehensive vocal repertoires have been described in a variety of different species (reviewed by BOINSKI *et al.* 1999, TEIXEIRA *et al.* 2019).

So far, acoustic parameters in animals have mainly served as indicators of negative affective states that were induced through physical pain, stress or isolation (e.g., MORTON & GRIFFITHS 1985, WEARY *et al.* 1997). However, recent studies have described clear acoustic correlates of positive affective states e.g., in rats (PANKSEPP & BURGDORF 2003), goats (BRIEFER *et al.* 2015), horses (STOMP *et al.* 2018), African elephants (SOLTIS *et al.* 2011) and great apes (ROSS *et al.* 2009). In addition, for some species, such as beluga whales (CASTELLOTE & FOSSA 2006), bottlenose dolphins (LIMA *et al.* 2017) and tufted capuchins (BOINSKI *et al.* 1999) previous studies already demonstrated that conclusions on stress response and activity budget could be drawn based on the rate of specific call types.

Most importantly, several studies showed that females' vocalisations during oestrus can be used as distinct indicators of their fertile phase, as they might utter specific advertisement calls (e.g., BUESCHING *et al.* 1998, DE HAAS VAN DORSSER *et al.* 2007), exhibit changes in the acoustic structure of calls (e.g., SEMPLE *et al.* 2002, SOLTIS *et al.* 2005), or increase the call rate (e.g., CHARLTON *et al.* 2010, LEONG *et al.* 2003, WIELEBNOWSKI & BROWN 1998). Overall, these findings strongly suggest that vocalisations should be included in the monitoring concepts for captive animals, as they contain valuable information – not only on the welfare but also on the reproductive state of the animals (MCLOUGHLIN *et al.* 2019).

Finally, even though the concept of bioacoustic monitoring is fairly straightforward, the practical applicability does not only require technical equipment and know-how but also time for coding and classification of vocalisations, which is still the most common technique despite the progressing automation of acoustic data analyses.

1.2.4 | Monitoring strategies in comparison

In general, zoos commonly combine different monitoring techniques. For instance, in order to obtain a full picture, observations of behaviour and vocalisation might be complemented with hormone analyses (WHITHAM & WIELEBNOWSKI 2013). All methods have different kinds of requirements and hence, different advantages over one another (*Table 1.1*). For instance, hormone monitoring only provides results retrospectively, as it requires time to collect a sufficient number of samples for a reliable representation of a hormone profile as well as to conduct the analyses in the lab, while behavioural and vocal indicators can be identified

and evaluated immediately. However, they require persistent and time-consuming recording beforehand in order to allow reliable conclusions, as irregular or only short observations could result in discreet or brief, but no less relevant, behaviours or calls passing unnoticed.

Table 1.1: Characteristics of the three different animal monitoring techniques.

	Behavioural monitoring	Endocrine monitoring		Bioacoustic monitoring
		Blood samples	Faecal samples	
Invasiveness for animal	✗	✓	✗	✗
Equipment	(✓)	✓	✓	✓
External expertise	✗	✓	✓	(✓)
Data analysis	(✓)	✓	✓	(✓)
First conclusions	immediately	retrospectively		immediately

✓: required, (✓): required for in-depth analysis, ✗: not required

A decisive criterion for choosing between the monitoring approaches is certainly its practicability for the animal keepers, who are responsible for the animals in question. The requirements for a suitable monitoring approach might therefore be simplicity, directness and compatibility with their work routine as well as the accuracy of the obtained information. Thereby, especially short-term decisions such as separating or uniting a breeding pair would require indicators that are intuitive and immediate, such as specific behavioural patterns or call types, and reflect the endocrine state of the animals at the same time.

In order to identify indicators that take the results of all three methods into account it is necessary to synchronise the techniques systematically and knowledgeably (WHITHAM & WIELEBNOWSKI 2013). Conclusively, potential discrepancies between the findings could be indications of other factors taking effect on e.g., the hormone-based but not the behaviour-based parameters, or the other way round. Abnormalities or disorders in the natural behaviour or the physiological processes of an animal could be identified in this process.

This information is especially crucial for threatened animal species, which on the one hand rely heavily on *ex situ* conservation but on the other hand do not seem to be able to reach a self-sustaining population stability in captivity. One of these species is the white rhinoceros that demonstrably endures reproductive impairments despite long-standing research and husbandry, as well as intensive breeding efforts (REID *et al.* 2012, ROTH 2006, SCHWARZENBERGER *et al.* 2003). Previous studies have repeatedly described that the reproductive issues, comprising physiological as well as external factors, are specifically pronounced in the F1-generation of white rhinoceros females, while the F0-generation has proven to be predominantly fertile (SWAISGOOD *et al.* 2006). Conclusively, a multidisciplinary approach to reproductive monitoring in this species could provide indications of determining factors for successful breeding in captivity.

1.3 | The white rhinoceros (*Ceratotherium simum*)

The white rhinoceros (*Ceratotherium simum*) is a large-bodied disperse living grazing mammal that is divided into two subspecies – the Northern (*Ceratotherium s. cottoni*) and the Southern (*Ceratotherium s. simum*) white rhinoceros. The first subspecies is considered „critically endangered“ with the only two remaining representatives living in the Oj Pejeta reserve, Kenya (EMSLIE 2021a, OJ PEJETA CONSERVANCY 2018). The latter one is categorised as “near threatened”, experiencing a decreasing and therefore fragile population trend due to continuing poaching threat, prolonged draught periods and political inconsistency (EMSLIE 2021b). After almost going extinct at the end of the 19th century (OWEN-SMITH 1975), careful repopulation and reintroduction measures led to an increase of approximately 18,000 Southern white rhinoceroses (as of 2018) that are presently found in open grassland in bushveld savanna habitats across the Southern African continent (Botswana, Mozambique, Namibia, South Africa, Zimbabwe, Kenya) (EMSLIE 2021b). No confirmed free-ranging population of the Northern white rhinoceros can be found in any of the original regions of occurrence such as Congo, Sudan or Chad ever since 2006 (EMSLIE 2021a).

1.3.1 | The social ecology

The megaherbivore has a social organisation that is atypical for the *Rhinocerotidae* family: Unlike the other four rather solitary living rhinoceros species the white rhinoceros displays a so-called semi-social lifestyle. Adult females with and without calves and subadults of both sexes form temporary or even more persistent groups consisting of up to six individuals, whereas males become strictly solitary as soon as they attain socio-sexual maturity at an age of ten to twelve years (OWEN-SMITH 1975, PENNY 1987, PIENAAR 1994, SHRADER & OWEN-SMITH 2002).

The home ranges of females are usually significantly larger than the ones of the adult males and often overlap with several male territories (PENNY 1987, PIENAAR 1994, SHRADER & OWEN-SMITH 2002). Although white rhinoceroses don't have a restricted mating season, some studies suggest that they show a seasonal peak in mating, starting at the beginning of the rainy season with the flush of nourishing vegetation (KRETZSCHMAR *et al.* 2004, PENNY 1987, PIENAAR 1994, VERVERS *et al.* 2017). Territorial bulls and cows only associate for a breeding period of a few weeks during the cow's most fertile cycle phase. During this time, the bull follows a single cow protractedly, trying to stop her from evading into a neighbouring territory (OWEN-SMITH 1975, PIENAAR 1994). Although there is some evidence that free-ranging white rhinoceros females might need an external stimuli, such as contact with a dominant bull to

ovulate (VAN DER GOOT *et al.* 2015), the white rhinoceros has been considered to be a spontaneous ovulator so far, which means females ovulate at a set interval irrespective of the presence of a male (ROTH 2006, SCHWARZENBERGER & BROWN 2013). In the wild a typical cycle interval appears to last around one month (SKINNER & CHIMIMBA 2005, VAN DER GOOT *et al.* 2015).

After a gestation period of about 16 months the cow gives birth to a single offspring - the first time at an age of about six to seven years (ESTES 1991, OWEN-SMITH 1973, PIENAAR 1994). Though the infants already start to graze a few months after birth, they are nursed for up to 18 months with a declining nursing frequency after about one year (ESTES 1991, OWEN-SMITH 1973). When the offspring reaches the age of about two years, the cow is usually ready to give birth to her new calf. Shortly before calving, cows reject their previous offspring, seek seclusion and invest their parental care in the new infant all along (OWEN-SMITH 1973, PENNY 1987).

1.3.2 | The main communication modalities

As white rhinoceroses have a relatively poor eyesight (OWEN-SMITH 1975), they greatly rely on olfactory cues usually transmitted by communal dung piles for recognition and territory marking (CINKOVÁ & POLICHT 2015, MARNEWECK *et al.* 2017, MARNEWECK *et al.* 2018). In addition, white rhinoceroses are considered to be highly vocal as well (ESTES 1991, PENNY 1987) and primarily use this form of communication to coordinate consortship behaviour and mother-infant relationships. Their vocal repertoire has been characterised by Owen-Smith (only onomatopoeic, 1973) and Policht *et al.* (2008), who described ten and eleven different call types and their behavioural contexts respectively, interestingly corresponding in only five of them. Additionally, the vocal repertoire of juveniles has been recently described by Linn *et al.* (2018), demonstrating that vocal production and usage are innate in the white rhinoceros and that some call types are uttered exclusively by juveniles.

While it has been suggested that white rhinoceroses might produce not only audible but also infrasonic vocalisation (VON MUGGENTHALER *et al.* 1993), to date there is no reliable information on specific call types containing infrasonic components. Finally, two studies by Cinková and Policht (2014, 2016) stated the existence of individual signatures in the vocalisation of white rhinoceroses on the basis of contact Pant calls, allowing information on sex, age and subspecies of the sender to be indicated.

1.3.3 | Captive breeding of white rhinoceroses

The EEP for the white rhinoceros was established in 1992 and is currently led by Safaripark Beekse Bergen (VERSTEEGE 2020). Even though the demographic recordings of the studbook registered a population growth over the last 25 years, reporting a current state of approximately 340 individuals in total, animal imports from the wild were still conducted (VERSTEEGE 2020). Therefore, a steady self-sustainable population of the captive Southern white rhinoceros in Europe can still not be declared (HERMES *et al.* 2004, SWAISGOOD *et al.* 2006, VAN DER GOOT *et al.* 2015, VERSTEEGE 2018). In fact, under current demographic parameters a recent population viability analysis projected a decline of 2% annually for the European captive population, listing the low proportion of females calving as the main factor (SCOTT 2020).

The findings of various research projects point to multifactorial underlying causes that eventually lead to the stagnant reproductive rate in captive white rhinoceroses. Physiological abnormalities, including prolonged cycle lengths of two months (BROWN *et al.* 2001, PATTON *et al.* 1999, SCHWARZENBERGER *et al.* 1998) as well as irregular or fully absent ovarian activity (reviewed by ROTH *et al.* 2018), have been repeatedly described in females. Further studies stated that prolonged exposure to sex steroids in non-reproducing yet continually cycling females evidently leads to a shortened reproductive life-span compared to the one in the wild (HERMES *et al.* 2004). This asymmetric reproductive aging process eventually favours the development of genital pathologies such as endometrial hyperplasia, leiomyomas, ovarian cysts or hydromecumetra that increase with age (HERMES *et al.* 2006). Moreover, male infertility, mainly characterised by inadequate sperm quality (HERMES *et al.* 2005), is considered a major problem and cause for the low reproductive rate as well (SCHWARZENBERGER *et al.* 2003). At the same time, significant effects of group composition on the social interactions, and therefore potentially on the reproductive success, have been described, suggesting that unfavourable dominance structures might even suppress fertility (CINKOVÁ & BIČÍK 2013, HERMES *et al.* 2005, METRIONE *et al.* 2007, SEROR *et al.* 2002).

Despite an interrelationship between the physiological and behavioural factors evidently emerging, to date they have not been systematically linked. Even though some studies have already accounted for the female acceptance of sexual behaviour from bulls as an additional indicator for oestrus (PATTON *et al.* 1999, RADCLIFFE *et al.* 1997, SWAISGOOD *et al.* 2006), the vast majority of endocrine studies have been conducted separately from detailed behavioural or bioacoustic analyses. Integrating these aspects could, however, provide evidence of how hormonal states might be reflected in behaviour and vocalisation, and thus lead to

comprehensive approaches that facilitate and complete the monitoring of the reproductive state in captive white rhinoceroses at the same time.

1.4 | Study aim

The present thesis aims to integrate approaches from three different disciplines (animal behaviour, endocrinology and bioacoustics), in order to present comprehensive insights into the social life and reproduction of captive Southern white rhinoceroses (*Ceratotherium simum simum*, SWR). For this purpose, data has been collected in 45 study animals, (16 ♂♂, 29 ♀♀), from eleven European zoos, over a total period of 12.5 months. Subsequently, the following three study objectives were set:

1) Investigation of social relationships based on vocal networks

Simultaneous audio- and video recordings of 30 SWRs in seven groups were conducted, focussing on the four most common call types: Snort, Hiss, Grunt and Pant. During vocalisations, the behavioural context and the nearest neighbours were determined, thereby considering neighbours at a proximity of ≤ 1 body length to the sender as potential receivers. Using this proximity-based approach directed call rates were calculated and effects of dyad and interaction type were identified. Moreover, vocal communication networks were constructed for each call type in order to analyse directionality and arrangement of vocal interactions.

2) Linking male faecal testosterone metabolites (fTM) with behaviour and vocalisation during female receptive period

Faecal samples of 16 SWR males, aged between one and 44 years, were collected and fTM levels were determined. Additionally, simultaneous audio- and video recordings were taken of five of the study males that were both, sexually mature and had full contact with receptive females. First of all, the development of fTM levels across different age classes was established. Subsequently, the effect of the females' receptive and non-receptive period on male fTM levels was determined. Finally, in order to investigate male behavioural and vocal indicators of female receptivity, the development of inter-sexual cohesion, affiliative, agonistic and olfactory behavioural rates as well as Pant and Hiss call rates was compared between the females' receptive and non-receptive periods.

3) Linking female behaviour and vocalisation with faecal progesterone and oestrogen metabolites (fPM and fEM) during receptive period

Faecal samples of 28 adult SWR females were collected and fPM and fEM were determined. Based on the fPM profiles hormonal oestrus could be detected in seven study females. In addition, simultaneous video- and audio recordings were taken of eight of the study females that accepted sexual behaviour from adult males, thereby indicating their receptivity. In these females, the coincidence of receptivity and hormonal oestrus was investigated and indicators of receptivity were examined. In order to do so, the rates of inter-sexual cohesion, affiliative, agonistic and olfactory behaviour as well as Pant, Hiss and Grunt call rates and fPM and fEM concentrations were compared between receptive and non-receptive periods.

2

Study I

Proximity-based vocal networks reveal social relationships in the Southern white rhinoceros

Jenikejew J¹, Chaignon B², Linn S³ & Scheumann M¹ (2020): Proximity-based vocal networks reveal social relationships in the Southern white rhinoceros. *Scientific Reports* 10, 15104.

<https://doi.org/10.1038/s41598-020-72052-0>

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JJ, BC and SL collected the data. JJ and BC performed the video analysis. JJ and BC performed the data analysis. JJ wrote the manuscript, prepared all figures and is the corresponding author. MS raised funding, designed and supervised the experimental study and data analysis.

2.1 | Abstract

Vocal communication networks can be linked to social behaviour, allowing a deeper understanding of social relationships among individuals. For this purpose, the description of vocal dyads is fundamental. In group-living species, this identification is based on behavioural indicators which require a high level of reactivity during social interactions. In the present study, we alternatively established a proximity-based approach to investigate whether sex-specific differences in vocal communication reflect social behaviour in a species with rather loose social associations and low levels of reactivity: the Southern white rhinoceros (*Ceratotherium simum simum*).

We performed audio- and video recordings of 30 captive animals from seven groups. Vocal networks for the four most common call types were constructed by considering conspecifics at close distance (≤ 1 body length) to the sender as potential receivers. The analysis of the resulting unidirectional structures showed that not only the sex of the sender but also the sex of the potential receiver, the quality of social interactions (affiliative or agonistic) as well as association strength predict the intensity of vocal interactions between group members.

Thus, a proximity-based approach can be used to construct vocal networks providing information about the social relationships of conspecifics - even in species with loose social associations where behavioural indicators are limited.

3

Study II

The female effect – How female receptivity influences faecal testosterone metabolite levels, socio-positive behaviour and vocalization in male Southern white rhinoceroses

Jenikejew J¹, Wauters J², Dehnhard M² & Scheumann M¹ (2021): The female effect - How female receptivity influences faecal testosterone metabolite levels, socio-positive behaviour and vocalisation in male Southern white rhinoceroses. *Conservation Physiology* 9, coab026.

<https://doi.org/10.1093/conphys/coab026>

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JJ collected the data and the samples, performed the video and data analysis, wrote the manuscript, prepared all figures and is the corresponding author. JW and MD supervised the endocrine analysis. MS raised funding, designed and supervised the experimental study and data analysis.

3. 1 | Abstract

Testosterone is known to be essential for sexual maturation as well as for the display of behavioural traits linked to reproduction. At the same time, external factors such as the presence of receptive females may affect testosterone levels, stressing the hormone's substantial role in reproductive success. It is therefore of major interest to investigate the links between androgens, behaviour and the social environment especially in species that rely on a resilient reproduction rate, such as the white rhinoceros (WR).

We collected faecal samples of 16 male Southern WR (*Ceratotherium simum simum*) aged between 1 and 44 years from 11 European zoos. Audio- and video recordings were simultaneously taken of five of the study males that were sexually mature and had direct contact with receptive females. Our results showed a positive correlation of faecal testosterone metabolite (fTM) concentrations and progressing age up until adulthood followed by a decline in older males. While previous reproductive success did not show any effect on androgen levels, the access to receptive females resulted in higher fTM levels. Thereby, fTM concentrations remained at the same level regardless of the receptivity phase, while social cohesion with respective females, affiliative behaviour as well as call rates of Pant and Hiss distinctly peaked during the receptive compared to the non-receptive periods.

Conclusively, the immediate presence of receptive females poses a female effect that enhances the overall androgen levels in males and thus, might facilitate their reproductive success. However, androgens do not seem to be the main driver of behavioural changes during courtship or mating. By linking endocrinological and socio-behavioural factors we were able to provide an applicable basis for non-invasive monitoring of reproductive behaviour in male WR in captivity, thereby contributing to deeper understanding of potential reproduction impairments in a species whose population in captivity remains not fully self-sustaining.

4

Study III

Linking socio-sexual and vocal behaviour with faecal progesterone and oestrogen metabolite levels in Southern white rhinoceros females

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Chapter 4

Study III

Jenikejew J¹, Wauters J², Dehnhard M² & Scheumann M¹ (2021): Linking socio-sexual and vocal behaviour with faecal progesterone and oestrogen metabolite levels in Southern white rhinoceros females.

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4.1 | Abstract

Progesterone and oestrogen are the main gonadal steroid hormones that regulate the ovarian activity and induce the fertile oestrus period in females. The monitoring of this phase is particularly decisive for captive breeding and is commonly based on the observation of female behavioural patterns that demonstrably coincide with their hormonal oestrus.

However, in the white rhinoceros, a species that is well known for its impaired reproductive rate in captive conditions, the female behavioural and vocal indicators of receptivity have not been systematically investigated or linked to their hormonal states so far. In order to close this gap, we combined behavioural and acoustic recordings with the analysis of faecal progesterone and oestrogen metabolites (fPM and fEM, respectively) in 28 adult Southern white rhinoceros (*Ceratotherium simum simum*, SWR) females from eleven European zoos. For eight of the study females we were able to detect a receptive period indicated by their acceptance of sexual behaviour directed towards them by the bulls. The comparison of behaviour and vocalisation between receptive and non-receptive periods in these females demonstrated that particularly *presenting* and *marking* behaviour distinctly peaked during the receptive period, indicating the significance of olfactory signalling in female reproductive behaviour. Based on the analysis of fPM profiles, we were able to identify different reproductive states (oestrous, anoestrous, pregnant) in the study females. In contrast, fEM profiles proved to be unsuitable for the detection of ovarian activity. For the majority of the females a coincidence of their receptive period and the hormonal oestrus, indicated by a nadir in fPM levels, could be detected. Conclusively, this study revealed a comprehensive behavioural repertoire that reflects the hormonal oestrus in SWR females and can therefore be reliably used for non-invasive reproductive monitoring.

4.2 | Introduction

Sexual behaviour is an entirely social behaviour and can be found across all living species. Encompassing all behavioural patterns that lead up to and involve mating, sexual behaviour is inevitable for reproduction and hence, for the survival of the species. In females, sexual behaviour mainly comprises the components with which they initiate copulation and respond to sexual initiations of the mating partner (NELSON 2011). These behavioural patterns are largely universal among mammals and include the seeking of vicinity of males, adopting species-specific coital postures or gestures as well initiating body contact to the male (BEACH 1976). In most, but not all mammalian species, sexual behaviour is restricted to the oestrus phase, as it signals the female's receptivity to the male, enabling mating only when the female

is on her peak of fertility (JENNINGS & DE LECEA 2020). Outside of this period, females usually reject the males.

In mammals, in both sexes sexual behaviour is largely regulated by gonadal steroid hormones (JENNINGS & DE LECEA 2020). While in males the main steroid hormone is testosterone, in females the ovarian steroids progesterone and oestrogen are known to modulate the endocrine cycle (JENNINGS & DE LECEA 2020). Starting with puberty, sexually mature females experience regular cycles of alternating rises of oestrogen and progesterone as a response of the interplay of hormones produced by the hypothalamus, pituitary and ovaries (HOBEIKA *et al.* 2020). Generally, the oestrous cycle is subdivided into the follicular and the luteal phase. During the follicular phase oestrogen levels rise in parallel to the growing follicles, eventually stimulating the secretion of follicle-stimulating hormone (FSH) and mainly luteinizing hormone (LH), resulting in the so-called LH surge (HOBEIKA *et al.* 2020). Shortly thereafter, the oocyte is released from the dominant *graafian* follicle into the fallopian tube, a process known as ovulation (BROWN 2018). With the subsequent onset of luteal phase, the ovulated follicle is transformed into the *corpus luteum*, which begins to secrete high levels of progesterone, preparing the oestrogen-primed endometrium for potential implantation (BROWN 2018). In case of successful fertilisation, the progesterone-dominated phase is prolonged throughout the gestation period until shortly before parturition (MEINECKE *et al.* 2010). In case pregnancy does not occur, the *corpus luteum* regresses by the end of the luteal phase, inducing a new cycle (BROWN 2018).

For a wide variety of different mammalian taxa, the proportion of female reproductive hormones during different oestrous phases have been directly linked to the occurrence of sexual behaviour. For instance, female gorals display sexual behaviour, most notably lifting up the tail, during the time of elevated oestrogen levels (KHONMEE *et al.* 2014). In goats, female-specific oestrus behaviour such as decreased appetite, restlessness, increased vocal activity, frequent urination and accepting mounting behaviour of males was clearly associated with peaking oestrogen levels (SANKARGANESH *et al.* 2014). Similarly, increased vocalisation and rolling correlated positively with oestrogen levels in Asiatic lionesses (UMAPATHY *et al.* 2007), just as in clouded leopard females increased oestrogen concentrations were associated with behavioural oestrus, entailing decreased food intake, lordosis posture and increased affiliative behaviour (BROWN *et al.* 1995). In primates the most fertile phase has been correlated with distinct display of sexual behaviour, peaks in oestrogen as well as a nadir in progesterone levels (ENGELHARDT *et al.* 2005).

This kind of hormonal and behavioural concurrence is of particular interest for captive breeding, as it entails crucial information on the most reasonable time point for mate pairing (GRAHAM 2004, LINDBURG & FITCH-SNYDER 1994). Especially in solitary species staging of mating can only occur when it can be ensured that the female will accept the sexual interactions of the male, which can only be specified by clear behavioural indicators. For instance, in giant pandas increased scent marking and bleat vocalisations are well established oestrus indicators in females (KLEIMAN 1983), while in cheetahs male sexual arousal (penile erections and stutter-barking) as a reaction to olfactory cues of oestrous females has been successfully implemented as a signal for pairings (LINDBURG *et al.* 1993). However, in order to correctly interpret the behavioural indicators of receptivity, it is necessary to not only know the entire behavioural repertoire but also to being able to align it with the hormonal states of the females (WHITHAM & WIELEBNOWSKI 2013). To date, this kind of relationship is still not fully understood in the majority of mammalian species (GANSWINDT *et al.* 2012).

In this respect, the white rhinoceros (*Ceratotherium simum*, WR) is a special case, as particularly the female's role in the reproduction of this megaherbivore species received major interest in recent years, with studies mainly addressing the issue of the sluggish reproduction rate in captivity (HERMES *et al.* 2004, SWAISGOOD *et al.* 2006, VAN DER GOOT *et al.* 2015). There have been many endocrinological investigations that repeatedly pinpointed the problem to aberrant ovarian activity in the majority of the females in reproductive age (reviewed by ROTH *et al.* 2018), partly attributing it to unnaturally long nulliparous phases that are observed in this species and the subsequent pathologies that come with that (HERMES *et al.* 2004, HERMES *et al.* 2006). However, so far these studies have carried out the endocrine investigations without considering systematic behavioural analyses.

Commonly, the display of mating has been used as behavioural indicator in previous studies, as it is known to coincide with the female oestrus (BROWN *et al.* 2001, PATTON *et al.* 1999, SWAISGOOD *et al.* 2006). Moreover, Radcliffe *et al.* (1997) also included accepting male sexual behaviour as well as urine squirting and lifting up the tail as typical oestrus behaviour when evaluating the reproduction of WR females. Nonetheless, these behavioural indicators have never been empirically proven and it has not been examined if there might be other indicators as well and how they develop throughout the female oestrous cycle and what might affect them.

In the wild, WR males are known to play the active role and initiate courtship and mating by approaching oestrous females and guarding them for several weeks during their most fertile phase (OWEN-SMITH 1975, PENNY 1987). A recent study clearly demonstrated that also in

captivity WR males approach and follow the female during her receptive period, while distinctly displaying their motivation by uttering the contact call Pant (JENIKEJEW *et al.* 2021). Besides, males spent an increased amount of time close to the female and displayed higher rates of affiliative behaviour before eventually start mounting (JENIKEJEW *et al.* 2021). So far, however, female sexual behaviour in WR has been less extensively studied. While it is well established that they only tolerate males' sexual interactions during their receptive phase (OWEN-SMITH 1988, OWEN-SMITH 1975), it is not known if they also use the Pant call in the socio-sexual context or if they prefer other communication modalities to signal their receptivity.

Accordingly, the present study aimed to integrate the different approaches of behavioural and vocal analyses as well as endocrinology to create a comprehensive picture of reproduction in WR females and by doing so, provide the foundation for an accessible non-invasive monitoring tool in captivity. In order to do so, we investigated the development of female behavioural and vocal parameters during the period when they accepted sexual interactions from males, to emphasise potential indicators of receptivity that would distinctly peak. Subsequently, we analysed faecal progesterone and oestrogen metabolite (fPM and fEM, respectively) levels to determine the ovarian cycle. Finally yet importantly, we examined if the hormonal oestrus of the cyclic females temporally coincided with their receptive period.

4.3 | Methods

4.3.1 | Study sites and animals

Overall, data was collected from 28 adult female Southern white rhinoceroses (*Ceratotherium simum simum*, SWR) at eleven European zoological institutions. Study females were kept in either one of five group categories varying in size and composition, consisting of 1) one adult bull, at least two adult females and at least one juvenile, 2) one adult bull and at least two adult females, 3) one adult bull and one adult female, 4) three adult females and at least one juvenile or 5) four adult females, two subadults and three juveniles (*Table 4.1*). Correspondingly, study females that were housed in direct contact with an adult bull (category 1-3) were further classified according to whether or not they accepted sexual behaviour from the bull during the observation period (*Table 4.1*).

4.3.2 | Vocal and behavioural data collection

Over an average period of 32 days, simultaneous acoustic and behavioural recordings were taken of all individuals in the groups using focal animals sampling (ALTMANN 1974). Each

focal animal was observed for ten minutes per session, resulting in 20 to 40 minutes daily observation time randomly distributed between 8 am and 6 pm.

Table 4.1: Information on study females during observation period.

Group categories: **1** - one adult bull, at least two adult females and at least one juvenile, **2** - one adult bull and at least two adult females, **3**- one adult bull and one adult female, **4** - three adult females and at least one juvenile, **5** - four adult females, two subadults and three juveniles. Detection of hormonal cycle only possible when study females were sampled on \geq three days/week.

ID	Zoo	Sampling year	Age [yrs]	Group category	Accepted sexual behaviour	Sampled period [days]	No. of daily samples	Hormonal cycle
Amelie	Osnabrück	2014	7	2	Yes	31	29	No
Marsita	Osnabrück	2014	9	2	Yes	29	10	?
Lia	Osnabrück	2014	12	2	No	30	10	?
Chris	Augsburg	2014	9	2	Yes	56	53	Yes
Kibibi	Augsburg	2014	9	2	Yes	56	53	Yes
Baby	Augsburg	2014	43	2	No	56	47	Yes
Shakina	Dortmund	2014	9	4	-	28	26	No
Jasira *	Dortmund	2014	9	4	-	26	24	No
Natala	Dortmund	2014	30	4	-	28	25	No
Temba	Erfurt	2015	17	2	No	40	18	No
Numbi	Erfurt	2015	18	2	No	40	18	No
Uzuri	Hodenhagen	2015	10	1	No	32	8	?
Kianga	Hodenhagen	2015	11	1	No	30	9	?
Claudia	Hodenhagen	2015	17	1	Yes	34	7	?
Doris	Hodenhagen	2015	43	5	-	38	16	No
Cera	Gelsenkirchen	2015	21	2	No	25	15	No
Tamu	Gelsenkirchen	2015	23	2	No	25	15	No
Clara	Schwerin	2018	12	2	Yes	27	12	Yes
Karen	Schwerin	2018	15	2	Yes	29	18	Yes
Jane	Münster	2018	19	1	Yes	31	24	Yes
Vicky	Münster	2018	32	1	No	25	8	?
Yoruba	Amnéville	2018	11	4	-	31	22	No
Hekaw	Amnéville	2018	14	4	-	31	22	No
Lucy *	Amnéville	2018	16	3	No	31	22	No
Tala	Amnéville	2018	19	4	-	31	21	Yes
Jamala	Knuthenborg	2019	5	2	No	32	31	No
Bodil	Knuthenborg	2019	22	2	No	32	31	No
Sana	Planète Sauvage	2019	55	3	No	13	4	?

* Study female was pregnant during observation period

Video recordings were made using a digital camcorder (Sony DCR-SR36E, Sony Corporation, Tokyo, Japan). Audio recordings were made using a Sennheiser omni-directional microphone (Sennheiser MKH 8020, Sennheiser electronic GmbH & Co. KG, Wedemark-Wennebostel, Germany; flat frequency response from 10-20000 Hz \pm 5db) that was equipped with a wind shield and a boom pole. The microphone was connected to a digital recording device (Sound devices 702T State Recorder, Sound Devices LLC, Reedsburg, USA; frequency response: 10-40000 Hz; settings: 44.1 kHz sampling rate, 16 Bit, uncompressed .wav format).

In the further analyses, only the recordings of the study females that were housed together with an adult male and accepted sexual interactions (*head placing, mounting,*

copulation) from them during observation period were included (N=8, *Table 4.1*). A sequence of consecutive days (± 1) on which sexual behaviour towards a female was observed, was considered the female's receptive period (KOMERS *et al.* 1994, PATTON *et al.* 1999, SWAISGOOD *et al.* 2006). Non-receptive periods were defined as three and six days (± 1) before the first display of sexual behaviour as well as three and six days (± 1) after the last day of sexual behaviour display.

Overall, 75 hours of data were analysed: 20 hours at Zoo Osnabrück (April 2014), 20 hours at Zoo Augsburg (July/August 2014), five hours at Serengeti-Park Hodenhagen (April/May 2015), 20 hours at Zoo Schwerin (April/May 2018) and ten hours at Zoo Münster (July/August 2018).

4.3.3 | Vocal and behavioural analysis

Video recordings were synchronised with respective audio recordings and analysed using the *Observer XT* software (version 12, Noldus Information Technology, Netherlands (NOLDUS 1991)). The analysis was conducted by two different observers (BC: Osnabrück, Hodenhagen, Schwerin; JJ: Augsburg, Münster). The Cohen's Kappa coefficient was determined among the observers by comparing 15 pilot observations (total of 100 min). K values were ≥ 0.95 , indicating a high interrater reliability (LANDIS & KOCH 1977).

Vocalisations were detected by auditory identification and categorised according to literature (LINN *et al.* 2018, OWEN-SMITH 1973, POLICHT *et al.* 2008). For each vocalisation the respective call type, sender as well as potential receiver were noted (see JENIKEJEW *et al.* (2020) for detailed description). Analysis focused on Pant, Hiss and Grunt calls only, as a previous study demonstrated sex-specific differences in call rates, and hence a potentially relevant role in mating context for these call types (JENIKEJEW *et al.* 2020).

Behaviour was coded considering proximity measurements of the focal female to present group members, taking adult body length (2.5-3 metres: OWEN-SMITH 1973) as measuring unit. The duration each focal female spent in close proximity (≤ 1 body length) to each group member was noted.

For each focal female the occurrence of affiliative, aggressive and defensive interactions and the respective interaction partner as well as olfactory behaviour were noted (see ethogram in *Table 4.2*). Affiliative interactions included social exploration of the interaction partner as well as socio-positive behaviour and *presenting*. Aggressive interactions were coded when the focal female *displaced*, *attacked*, *chased*, *pushed* or *clashed horns* etc. with the interaction partner, whereas defensive interactions were coded when the focal female *avoided* or *escaped*

from the interaction partner. Olfactory behaviour comprised *marking* as well as *sniffing* and *flehming*.

Table 4.2: Ethogram of olfactory, affiliative and agonistic behaviour of captive Southern white rhinoceroses.

	Behaviour	Description
Olfactory	Marking	Focal animal urinates intermittently or spreads its defecation with its hind legs
	Sniffing	Focal animal explores ground/objects or urine/faeces by inclining towards it, "sliding" along the surface with the snout
	Flehming	Focal animal opens its mouth and curls back its upper lip exposing its upper gum while inhaling
Affiliative	Following	Focal animal moves after a conspecific while it changes the location
	Snout contact	Focal animal explores the body of another conspecific (except the snout) with its snout
	Social Flehming	Focal animal flehms while scenting a defecating/ urinating conspecific close by
	Naso-nasal sniffing	Focal animal contacts the nasal region of another conspecific with its own snout
	Ano-genital sniffing	Focal animal contacts the ano-genital region of another conspecific with its own snout
	Head placing	Focal animal lays its head on the back of another conspecific (<i>only for bulls</i>)
	Body contact	Focal animal touches or brushes another conspecific while moving with any part of its body (except snout) or rubs itself against a conspecific
	Presenting	Focal animal lifts up its tail while the bull is standing behind
	Mounting	Focal animal climbs with its forelegs on another conspecific (<i>only for bulls</i>)
Copulation	The animals mate: the bull inserts his penis into the cow	
Aggressive	Displace	Focal animal incites a conspecific to change its position/location after approaching or agonistic interaction
	Nodding	Focal animal swings its head back and forth
	Lifting	Focal animal lifts another conspecific's head or leg with its head/horns
	Staring	Focal animal is standing horn to horn in front of another conspecific with an uplifted
	Pushing	Focal animal presses any part of its body against another conspecific making it change the position/location
	Chasing	Focal animal <i>follows</i> another conspecific, which tries to keep the Focal animal at a distance, in a trotting manner
	Feigned attacking	Focal animal moves with a lowered head towards another conspecific and stops suddenly without causing body contact
	Attacking	Focal animal hits its horn against another conspecific
Horn clashing	Escalated confrontation following <i>Attacking</i> involving both animals hitting their horns against each other	
Defensive	Avoiding	Focal animal changes its position or location after being approached by a conspecific, agonistic interaction with or agonistic vocalisation from it
	Escaping	Focal animal moves away from a conspecific in a trotting manner after an agonistic interaction

4.3.4 | Faecal sample collection

Hormonal levels were measured non-invasively by analysing excreted hormone metabolites in the faeces. Individual faecal samples were collected on two to seven days weekly over an average period of 32 days (*Table 4.1*). To ensure a clear individual assignment and to collect the samples at an approximately same time of the day, faecal samples were collected in the morning after the study animals were housed separately during the night (BROWN *et al.* 2001, CARLSTEAD & BROWN 2005). When females were housed together with their calf, dung piles were distinguished based on the size of the faeces balls. In Zoo Schwerin faecal samples were collected immediately after defaecation, as the animals were not separated during the

night. Accordingly, the general time lag between defaecation and sample collection did not exceed 12h. Immediately after collection, samples were frozen and stored at -20°C until further analysis.

4.3.5 | Hormone metabolite determination

After defrosting, a representative subsample of 0.5 g was weighed from each faecal sample. Subsequently, 4.5 ml 90% methanol (MeOH) was added, followed by 30 min shaking. The mixture was centrifuged for 15 min at $1000 \times g$ (Rotanta 46RC, Hettich GmbH & co, Tuttlingen, Germany). An aliquot of 0.5 ml supernatant was then transferred to an Eppendorf vial and diluted 1:1 with distilled water to obtain a 45%-MeOH extract. Of this final extract, 20 μ l was used for each EIA well. This sample extract was combined with 100 μ l of enzyme label (1:2400 and 1:3200, for progesterone and estrogens respectively) and 100 μ l antibody (1:6000 and 1:80.000 for progesterone and estrogens respectively). After overnight incubation at 6-8°C on a shaking platform (Ika Vibrax VXR, IKA® - Werke GmbH & co KG, Staufen, Germany), the microtitre plates were washed four times (Hydrospeed, Tecan Group Ltd., Männedorf, Switzerland) before adding 150 μ l substrate buffer (TMB) for 40 min at room temperature, without shaking. The enzymatic reaction was stopped by adding 50 μ l 2M sulphuric acid to each well and the absorbance was subsequently measured at 450 nm (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland).

Progesterone was quantified by an EIA according to Dehnhard *et al.* (2008) using a commercial progesterone antibody raised in rats (Sigma P1922) and a 4-pregnen-3,20-dione-3-CMO-peroxidase label. The cross-reactivities to other steroids were as follows: 4-pregnen-3,20-dione (progesterone), 100%; 5 α -pregnan-3,20-dione (5 α -DHP), 76.8 %; 5 α -pregnan-3 β -ol-20-one (5 α), 5-pregnen-3 β -ol-20-one, 10.8%; 18.3 %; <0.1% for 5 β -pregnan-3 α -ol-20-one, 20 α -dihydroprogesterone, pregnandiol, 17 α -hydroxyprogesterone, testosterone, estradiol, and cortisol.

The inter-assay CV (9 assays), based on a low quality control sample (LQC) and high quality control sample (HQC), both fitting the linear range of the curve and run in duplicate, was respectively 10.3 and 7.4%. The intra-assay CV, determined on two biological samples including low and high concentration respectively (16 repeats in duplicate each), was 4.1% and 4.2%, respectively. The range of the calibration curve (standard progesterone) was 0.2-100 pg/20 μ l. The linear range, between B80 and B20, was determined between 1.8 and 20.0 pg/20 μ l. All EIA measurements were performed in duplicate with acceptance criteria of a coefficient of variation (CV) below 5%.

Oestrogens were quantified by an EIA according to Carnaby *et al.* (2012) using a polyclonal antibody raised in rabbits to 1,3,5(10)-estratrien-3,17b-diol-17-HS-BSA and a 1,3,5(10)-estratrien-3,17b-diol-17-HS-peroxidase label. The cross reactivities to oestrogens were as follows: 1,3,5(10)-estratrien-3,17b-diol (17b-E2), 100%; 1,3,5(10)-estratrien-3,17-one (estrone), 100%; 1,3,5(10)-estratrien-3,17a-diol (17a-E2), 66%; 1,3,5(10)-estratrien-3,16a,17b-triol (oestriol), 1.5%; and 0.1% for 19-nortestosterone, P4 and testosterone.

The inter-assay CV (7 assays), based on a low quality control sample (LQC) and high quality control sample (HQC), both fitting the linear range of the curve and run in duplicate, was respectively 5.73 and 12.74%. The intra-assay CV, determined on two biological samples including low and high concentration respectively (16 repeats in duplicate each), was 3.46% and 2.97%, respectively. The range of our calibration curve (standard 17b-E2) was 0.2-100 pg/20µl. The linear range, between B80 and B20, was determined between 0.95 and 15.11 pg/20µl. All EIA measurements were performed in duplicate with acceptance criteria of a coefficient of variation (CV) below 5%.

Progesterone metabolites were analysed in the faecal samples of all study females, while oestrogen metabolites were not analysed in the faecal samples of study females from *Dortmund* and *Knuthenborg* (Table 4.3). Hormone metabolite values were dated with a delay of one day based on a previous study by Hindle and Hodges (1990) that described a peak of metabolite concentrations in faeces 24 hours after the intravenous injection of radiolabelled oestradiol-17β and progesterone in Southern white rhinoceros females.

Definition of a hormonal cycle

A hormonal cycle was determined by assessing the dynamics of faecal progesterone metabolite (fPM) levels indicating a periodic pattern (BROWN *et al.* 2001, PATTON *et al.* 1999). For each study female that was sampled on at least three days weekly, a cycle was identified if the fPM values remained under baseline level ($\bar{x}-0.5\times SD$) for at least a week and were followed by a substantial rise and stayed above maximum level ($\bar{x}+0.5\times SD$) for at least three consecutive values, eventually returning back to baseline levels within one week.

4.3.6 | Statistical analysis

Statistical tests were calculated in *RStudio* (version 4.0.2, RSTUDIOTEAM 2016). The significance level was set at $p\leq 0.05$, $p<0.1$ was considered a statistical trend. Normal distribution of individual hormone metabolite levels as well as mean hormone metabolite levels was verified using the Kolmogorov-Smirnov test ('ks.test' function) and Q-Q plots. Residuals

were calculated for all LMEs ('resid' function) and subsequently verified for normality as well as for homogeneity of variances.

Differences across receptive and non-receptive periods

For the study females that were housed with adult bulls and were observed accepting sexual behavioural from them, thereby signalling their receptivity (N=8), the dynamics of behavioural and vocalisation rates as well as fPM and fEM concentrations were investigated over the course of the receptive and non-receptive period.

In order to quantify behaviour and vocalisation, daily proximity, olfactory, interaction as well as call rates were calculated for each focal female. Daily proximity rate was calculated for each one of the focal female-male dyads by dividing the duration the focal female spent in close proximity (≤ 1 body length) to the male by the total observation time of the focal female on that day. Daily proximity rate was indicated in minutes per hour, ranging from 0 to 60 minutes. Thus, a value of 60 indicated that the female-male dyad spent the full hour together, whereas a value of 0 indicated that the female-male dyad spent no time together. In doing so, we assessed the level of social cohesion between focal female and adult male. Daily interaction rates were calculated by dividing the number of a) affiliative, b) aggressive and c) defensive interactions of the focal female with the male by the total observation time of the focal female on that day. Daily olfactory rates (*marking, sniffing/flehming*) were calculated by dividing the number of displayed behaviour by the total observation time of the focal female on that day. Daily directed call rates were calculated for each call type by dividing the number of calls the focal female uttered to the male by the total duration the focal female spent in close proximity to the male on each observation day. Daily interaction, olfactory and call rates were indicated as number per hour.

Daily fPM and fEM concentrations as well as daily behavioural rates and call rates were determined for the oestrus period (*d0*) and non-oestrus periods (*d0+/-3, d0+/-6*). Mean values were calculated for each period including +/- 1 day.

Subsequently, we investigated whether the different periods (*d0-6, d0-3, d0, d0+3, d0+6*) had an effect on the mean values by calculating LMEs using the periods as predictor variable, while controlling for "zoo" as random factor. A significant effect was determined by the likelihood ratio test ('car' package, 'Anova' function). Multiple comparisons between the receptive period and the non-receptive periods were Bonferroni-Holm adjusted ('p.adjust' function, 'holm' method).

For behaviour and call types that were only rarely observed (defensive interactions, *marking*, *presenting*, Pant call rate, Grunt call rate), a chi-square test was calculated in *SPSS* (IBM CORP. 2019) in order to compare the number of individuals, which displayed the behaviour or uttered a call during the different periods (*d0-6*, *d0-3*, *d0*, *d0+3*, *d0+6*).

4.4 | Results

4.4.1 | Differences across receptive and non-receptive periods

Behaviour and vocalisation

The period of female receptivity proved to have a significant effect on social cohesion between the study females and the adult males ($p=0.002$, *Figure 4.1A*). Study females spent significantly more time in close proximity to adult males during the receptive compared to the non-receptive periods ($0.01 \leq p \leq 0.035$).

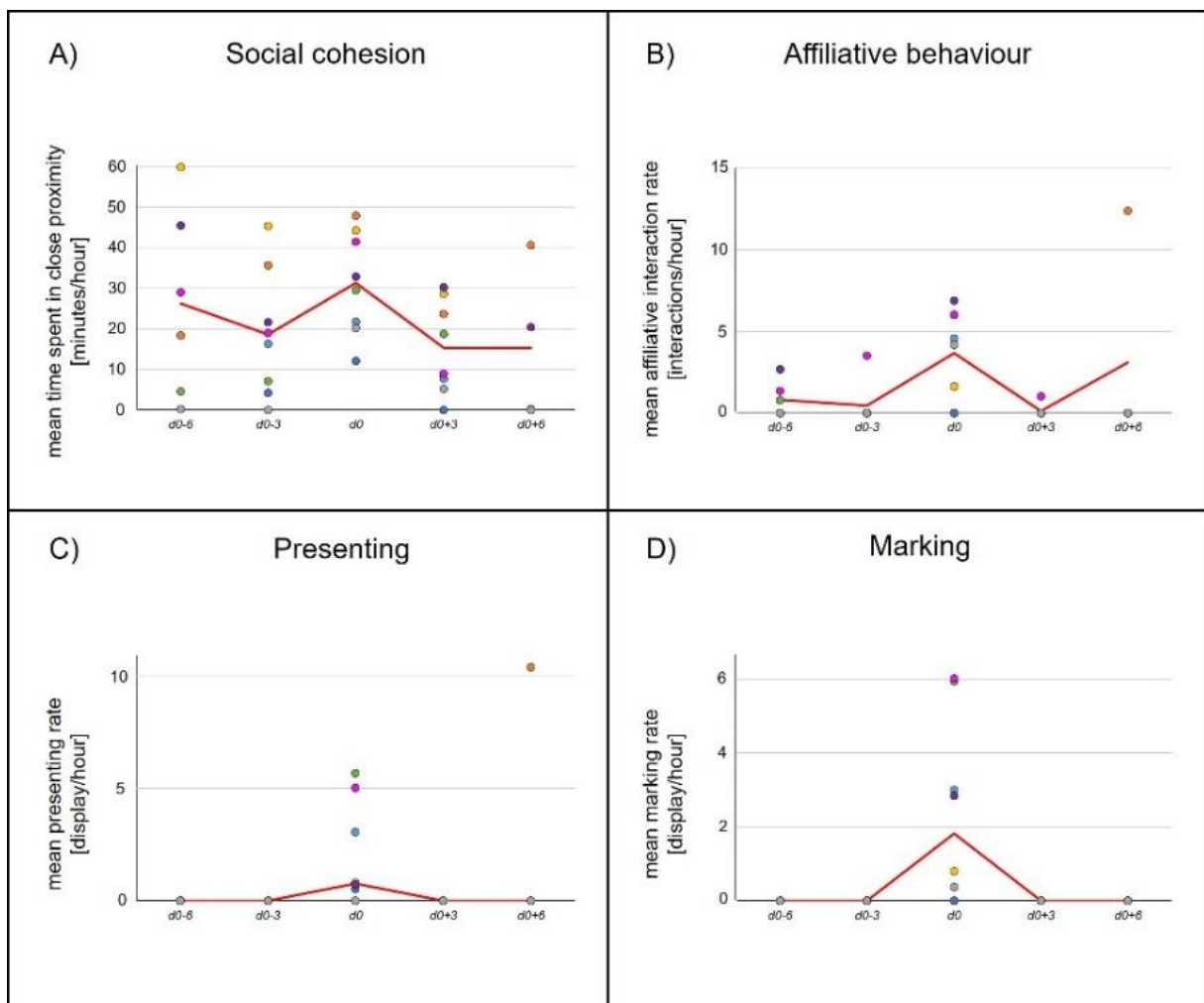


Figure 4.1: Mean values of **A)** time spent in close proximity to the adult male, **B)** affiliative interaction rate with adult male, **C)** lifting up the tail when close to the adult male and **D)** urinating intermittently. Each data point represents a study female during receptive (*d0*) and non-receptive (*d0±3/6*) periods. Red coloured lines represent the mean values (A+B) or median values (C+D) over all females.

Furthermore, the period of female receptivity proved to have a significant effect on female affiliative interactions towards males as well ($p=0.009$, *Figure 4.1B*). The pairwise comparisons revealed a significant difference between the receptive period and the non-receptive period for $d0+3$ ($p=0.040$). During the non-receptive periods before, affiliative interaction rates showed a statistical trend to be lower compared to the receptive period ($0.053 \leq p \leq 0.094$). Focusing on the affiliative behaviour of *presenting* in particular demonstrated that with the exception of one female this behaviour was displayed during the receptive period only ($\chi^2=20.938$, $df=4$, $p<0.001$, *Figure 4.1C*).

For the potential effect of receptive and non-receptive periods on aggressive interaction rates towards males a statistical trend was revealed ($p=0.094$). Further pairwise comparisons did not show any significant differences. For the defensive interaction rate no significant difference between the receptive and non-receptive period was found ($\chi^2=3.896$, $df=4$, $p=0.420$).

No significant effect of female receptivity on *sniffing* and *flehming* rate could be found in the study females ($p=0.244$). However, *marking* behaviour was significantly higher during the receptive period compared to the non-periods periods before and after, as the females displayed this olfactory behaviour exclusively during their receptive period ($\chi^2=24.440$, $df=4$, $p<0.001$, *Figure 4.1D*).

Even though female receptivity proved to have a significant effect on the Hiss call rate towards males ($p=0.021$), the further pairwise comparisons did not reveal any significant differences in Hiss call rate between the receptive period and the non-receptive period before and after. Similarly, no significant difference was found either for the Grunt call rate ($\chi^2=6.032$, $df=4$, $p=0.197$) or the Pant call rate ($\chi^2=6.906$, $df=4$, $p=0.141$). Regarding the Pant calls the statistical outcome may be attributed to the fact that only two out of eight females uttered this call type at all. However, the ones that did utter Pants, uttered them exclusively during the receptive period.

Faecal hormone metabolites

No significant effect of female receptivity on mean fPM or fEM concentrations ($p=0.131$ and $p=0.228$, respectively) could be found in the study females that were housed with adult bulls and were observed accepting sexual behavioural from them. Accordingly, there were no significant differences in mean faecal hormone metabolites between the receptive and non-receptive periods before or after.

4.4.2 | Hormonal cycles

Overall, for 21 of the study females a sufficient number of faecal samples was collected, allowing the detection of ovarian activity. Six of these study females signalled their receptivity towards the bull, eight did not accept sexual behaviour from the bull and seven were housed without direct contact with a bull.

A hormonal cycle could be determined in seven females. While in these cases fPM levels showed a specific pattern of a clear nadir of approximately one to two weeks, framed by fPM levels that were more than twice as high before and after, fEM concentrations did not show any specific patterns in their dynamics and fluctuated from day to day at approximately constant levels.

Of the six study females who indicated their receptivity by accepting sexual interactions from adult bulls, five were those who were identified to have had a hormonal cycle (example *Chris*, Figure 4.2A). One of them, however, was found to have had an irregular fPM profile that did not indicate a hormonal cycle (*Amalie*, Figure 4.2B).

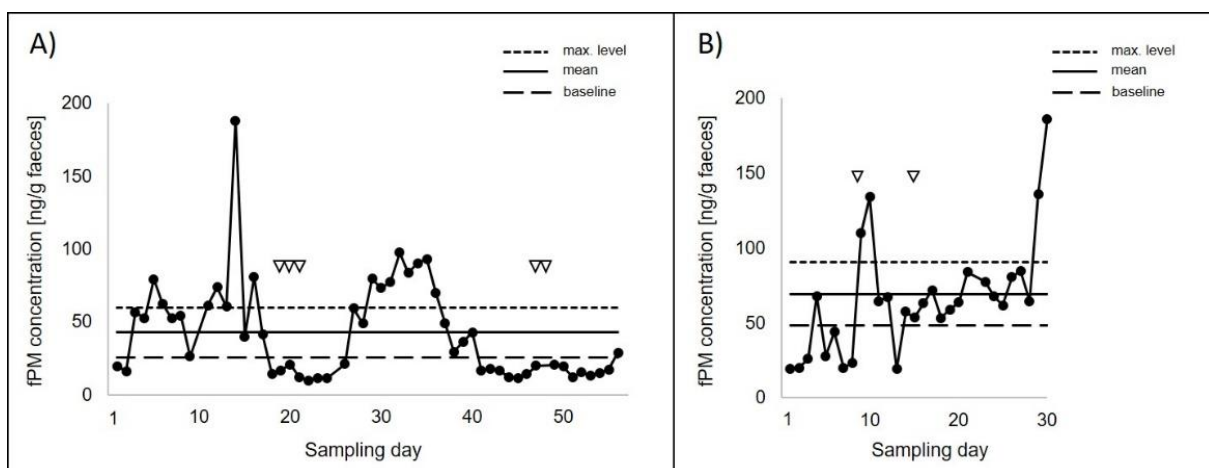


Figure 4.2: Concentrations of faecal progesterone metabolites (fPM) in Southern white rhinoceros females signalling receptivity.

Triangles represent acceptance of sexual behaviour from male, indicating female receptive period. Mean: \bar{x} of fPM levels over sampling period, max: $\bar{x} + 0.5 \times \text{SD}$, baseline: $\bar{x} - 0.5 \times \text{SD}$. **A)** Study female with regular oestrous cycle and coinciding receptivity. **B)** Study female with an irregular oestrous cycle and sporadic display of receptivity.

Of the eight study females that were kept with an adult male but did not indicate any receptivity, no hormonal cycle could be determined (example *Temba*, Figure 4.3A), except for one (*Baby*, Figure 4.3B). In addition, the hormone profile of one of the females showed particularly high fPM values compared to the others (*Lucy*, Figure 4.4): Approximately two weeks into sampling, her fPM levels doubled to more than 550 ng/g faeces within four days and remained elevated. It should be noted that this female mated two months before the

beginning of the data collection and based on weekly blood sample analysis pregnancy was confirmed by the zoo-veterinarian at the end of it.

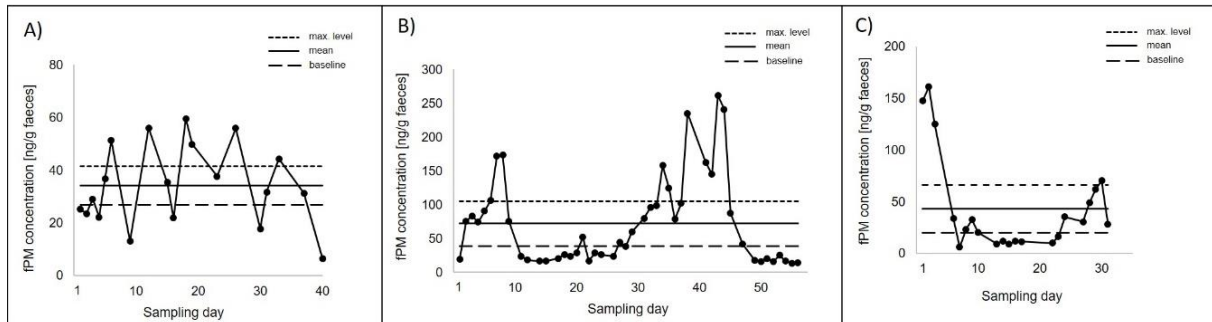
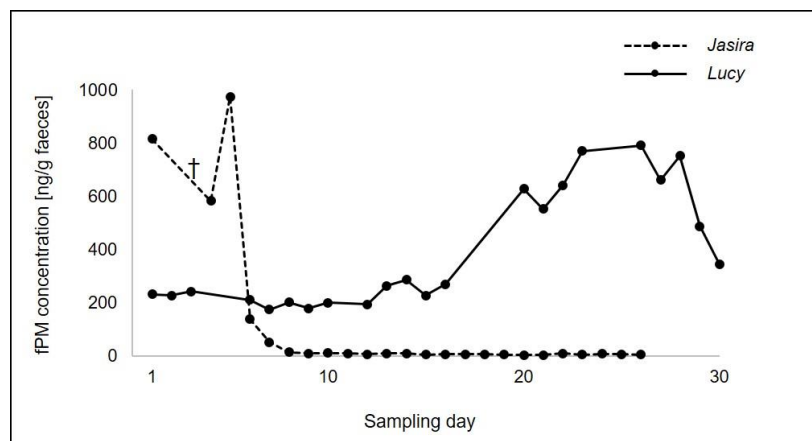


Figure 4.3: Concentrations of faecal progesterone metabolites (fPM) in Southern white rhinoceros females. Mean: \bar{x} of fPM levels over sampling period, max: $\bar{x} + 0.5 \times SD$, baseline: $\bar{x} - 0.5 \times SD$. **A)** Study female without a regular oestrous cycle and no display of receptivity while housed with adult male. **B)** Study female with a regular oestrous cycle but no display of receptivity while housed with adult male. **C)** Study female with a regular oestrous cycle while housed separately from adult male.

Finally, of the seven study females that were housed separately from the adult males, a hormonal cycle could be identified in one (*Tala*, *Figure 4.3C*). The hormone profile of another study female housed separated from an adult bull showed comparatively high fPM levels of about 800 ng/g faeces at the beginning of the sampling period that suddenly dropped to a one-hundredth of the maximal value and remained at a very low level of approximately 10 ng/g faeces (*Jasira*, *Figure 4.4*). This female endured a stillbirth and the dead infant was delivered by the attending veterinarian three days into sampling and only a few days away from estimated parturition.

Figure 4.4: Concentrations of faecal progesterone metabolites (fPM) in two pregnant Southern white rhinoceros females. *Jasira* during late pregnancy, stillbirth occurred on third sampling day (†). *Lucy* during early pregnancy, mating occurred two months before first sampling day.



4.5 | Discussion

The findings of the present study provide comprehensive insights into the interplay of behaviour, vocalisation and hormonal states for SWR females. Their receptive period indicated by accepting male sexual behaviour clearly coincided with distinct peaks in female affiliative

and olfactory behaviour. Furthermore, by analysing the profiles of fPM concentrations we were able to identify different reproductive states and demonstrate a coincidence between observed receptivity and hormonal oestrus for the majority of the females.

While female affiliative behaviour towards males as well as their rate of social cohesion with males generally peaked during receptivity, *presenting* and *marking* behaviour stood out specifically, as females displayed those almost exclusively during the time they accepted sexual interactions from males. In addition, Pant calls were uttered, even if only by very few females, exclusively during their receptive period, too. Hence, these findings not only provide first statistical evidence for female behavioural indicators of their receptivity described in previous studies (e.g., RADCLIFFE *et al.* 1997), but they also prove that olfactory communication is particularly crucial for signalling their reproductive state. While on the one hand, the female behavioural and vocal indicators differ from the ones of the males (JENIKEJEW *et al.* 2021), as the females' vocal activity did not peak during their receptive period, they also complement each and provide a complete picture of the courtship and mating ritual in white rhinoceroses. During their receptive period, females offer the males more opportunities to take in their olfactory cues by increasingly spraying urine and lifting up their tail. As soon as the males have absorbed the respective stimuli, they seek out the female and signal their interest by uttering the Pant call while approaching. These behavioural patterns correspond to the social organisation and spatial distribution of the animals in the wild (OWEN-SMITH 1988, OWEN-SMITH 1975, SHRADER & OWEN-SMITH 2002) and, interestingly, seem to be maintained in captive conditions as well.

In contrast, the female aggressive behaviour and the agonistic call types Hiss and Grunt towards the males did not change throughout the receptive and non-receptive periods. Even though we would have expected the aversive behaviour of the females to decrease during their receptive period so they would act less rejecting towards the bulls, it turned out that the rates of agonistic behaviour and vocalisations remained unchanged during receptivity and thus, only the affiliative and olfactory behaviour increased.

Regarding the development of fPM and fEM levels, no significant differences between the receptive period and the non-receptive period were found in this study, contrasting to behavioural rates. We would have expected a peak of fEM levels during the receptive phase or shortly before, indicating the imminent ovulation, but we could not confirm this. A possible explanation could be that fEM measures in WR have so far failed to create conclusive data and hence, provide a useful assessment tool for the follicular activity, as previously stated by several studies (BROWN 2018, BROWN *et al.* 2001, ROTH *et al.* 2018). According to Schwarzenberger

and Brown (2013) this is mainly attributable to the low quantities of oestrogens produced by the follicles in African rhinoceros species, as studies on Indian rhinoceros (*Rhinoceros unicornis*) females, having particularly large follicles, proved that faecal oestrogen measures in this species can be implemented to assess the cycle phase (SCHWARZENBERGER *et al.* 2000, STOOPS *et al.* 2004). Nevertheless, we expected that a higher sampling rate may have improved the accuracy of the assessment and indicated the volatile peak in oestrogen levels during the follicular phase. However, even sampling at a daily rate as in three of our oestrous study females did not result in any distinct oestrogen pattern that could have signalled a hormonal oestrus. Therefore, fEM profiles could not be used for the identification of ovarian activity in WR females.

As for the fPM levels, the lack of differences in concentrations between the receptive and the non-receptive period falls in line with the nadir of progesterone levels described for the follicular phase. In WR females, the increase in progesterone concentrations has been previously estimated to begin between six to nine days after ovulation (PATTON *et al.* 1999, RADCLIFFE *et al.* 1997, SCHWARZENBERGER *et al.* 1998). Hence, it is likely that we would have detected increasing progesterone levels if we have included a longer non-receptive period into our analysis. However, for half of the study females that signalled their receptivity, the subsequent observation period and thus the sampling period lasted less than a week. This means that for future studies a longer observation period should be targeted following the female oestrus to facilitate a comprehensive characterisation of the complete cycle. Nevertheless, these findings also provide additional support for the fact that there might be other hormonal messengers that elicit the behavioural changes during their receptive period in females.

By analysing the development of fPM concentrations throughout the observation periods, we were able to assign the hormonal profiles of 21 study females to different reproductive states, there being oestrous, anoestrous and pregnant states.

In five of the eight females that signalled their receptivity by accepting sexual interactions directed towards them by adult bulls, a hormonal cycle could be detected. In these five females, the receptive period temporally coincided with the nadir of the fPM levels and hence, with the follicular phase. This correspondence provides clear endocrine evidence that the behavioral indicators described above do in fact mark the fertile phase of the WR females. For two of the other females the sampling rate was not sufficient in order to reliably detect a hormonal cycle. One female, however, presents a noteworthy exception to the coincidence between the receptive period and the hormonal oestrus.

Even though the female in question clearly accepted sexual behaviour from the bull, her fPM profile did not reveal the expected cyclic pattern, as it was lacking of a nadir that would have lasted over several consecutive values and showed rather variable excretion instead. Therefore, it was not in accordance with what has been described as the definition of a hormonal cycle in white rhinoceroses by previous studies (BROWN *et al.* 2001, PATTON *et al.* 1999). This irregularity in ovarian activity was also reflected in the expression of the behavioural patterns that indicated her receptivity. While in the other study females the receptive period occurred accumulated over the course of a few consecutive days, the female in question displayed the behavioural indicators rather sporadically with several days between the occasions. Hence, we would need to carefully reconsider, if the displayed acceptance of sexual behaviour truly represented a receptive period in this female, as it was irregular and did not correspond to the normal case observed in this study.

On the one hand, this exceptional case demonstrates that the inconsistency between endocrine state and behavioural patterns can be used as a potential indicator for aberrations in the reproductive cycle of the females. On the other hand, it also gives rise to the question of whether there might be another driver for the behavioural changes in females than the metabolites analysed in this study. Findings from another study female point into the same direction, as we could describe a hormonal oestrus for her while she did not signal her receptivity towards the male. In view of the fact that she was one of the oldest study females it might be concluded that while aged females might still experience an endocrine cycle, they do not necessarily display the respective behaviour and hence, are not considered being reproductively active anymore. Therefore, further studies that would identify the potential hormone driver of behavioural changes during oestrus as well as characterise its function and development in different reproductive stages would be necessary.

Finally, the example of a study female with a hormonal oestrus but no access to an adult bull provides clear evidence that SWR females are spontaneous ovulators, as already described by Roth (2006). Nevertheless, male stimulation still appears to be required in order to show the full female behavioural repertoire that signals receptivity, as study females with direct contact to bulls did not only accept their sexual interactions but also displayed other behavioural and vocal indicators, such as *presenting*, *marking* and *panting*, which were completely absent in the female without male contact. Similar links between male stimuli and female sexual behaviour have been shown in other mammalian species that are spontaneous ovulators. In rats (MCINTOSH *et al.* 1978) and mice (HAMMERSCHMIDT *et al.* 2009) male ultrasonic vocalisations elicited approaching and solicitation behaviour in the females, while in sheep and goats

(reviewed by DELGADILLO *et al.* 2009) as well as in many primate species (reviewed by ROOKER & GAVRILETS 2020) the mere introduction of a male induces receptive behaviour in the females. However, this type of behavioural change in females, triggered by the absence or presence of male stimuli, has not yet been studied in WR. Hence, future studies focusing on the comparison between females with male contact and without could reveal further information about a possible male effect in this species.

Finally, the fPM profiles of the pregnant study females proved to correspond with previous descriptions of hormonal profiles in pregnant WR, describing elevated faecal pregnane levels with a steep increase exceeding luteal concentrations occurring around three months post-conception and remaining at high levels up until shortly before parturition (HILDEBRANDT *et al.* 2007, PATTON *et al.* 1999, PENNINGTON *et al.* 2020, VAN DER GOOT *et al.* 2013). One of the two pregnant females endured stillbirth during late pregnancy while the other one was at the early stage. Reportedly, the latter one gave birth to a female calf in November 2019, which corresponds the estimation of her being approximately two to three months pregnant during our observation period in September/October 2018. We do not have further information on what maternal or embryonic factors might have caused the embryo loss in the first female. Overall, these particular fPM profiles not only provide an adequate illustration of fPM profiles in pregnant WR females but also lend strong support for the biological validation and reliability of the hormone assay implemented in this study.

In conclusion, our results provide a comprehensive list of the behavioural indicators that female SWR use in order to signal their receptivity. Thereby, it is not only the passive behavioural patterns such as allowing a male to approach and ultimately accepting sexual interactions from him that indicate the receptive phase, but also active behaviour that is specifically related to olfactory communication such as *presenting* and *marking*. Furthermore, we confirmed that faecal progesterone metabolite but not oestrogen metabolite levels are suitable for describing ovarian activity in SWR females. Using the profiles of fPM concentrations that are characteristic for an ovarian cycle, we were able to identify different reproductive states of the females during the sampling period: pregnant, oestrous and anoestrus. For the majority of study females that indicated their receptivity by accepting sexual behaviour from bulls, the receptive period coincided with the hormonal oestrus, which additionally confirms the behavioural indicators detected in this study on endocrinological level.

4.6 | Appendix

Table 4.3: Hormone parameters of faecal progesterone metabolites (fPM) and faecal oestrogen metabolites (fEM) in Southern white rhinoceros females. SD = standard deviation.

ID	Zoo	fPM				fEM			
		Mean [ng/g]	Min [ng/g]	Max [ng/g]	SD [ng/g]	Mean [ng/g]	Min [ng/g]	Max [ng/g]	SD [ng/g]
Amelie	Osnabrück	69.18	19.16	185.73	42.15	7.40	2.95	14.63	2.63
Marsita	Osnabrück	74.83	23.07	132.93	29.67	11.44	8.98	17.00	2.56
Lia	Osnabrück	33.42	5.02	60.37	21.72	7.07	4.94	12.35	2.19
Chris	Augsburg	42.58	9.72	187.92	33.72	6.45	3.66	18.12	2.21
Kibibi	Augsburg	75.77	8.21	384.15	71.47	5.23	1.56	11.47	2.19
Baby	Augsburg	71.94	13.00	261.90	65.85	7.01	4.21	16.78	2.27
Shakina	Dortmund	7.20	2.88	13.36	2.93	-	-	-	-
Jasira *	Dortmund	113.72	4.32	975.00	269.55	-	-	-	-
Natala	Dortmund	11.62	4.29	18.98	4.00	-	-	-	-
Temba	Erfurt	34.19	6.55	59.57	15.41	11.51	6.84	24.37	3.68
Numbi	Erfurt	29.59	8.49	63.42	14.35	7.12	5.39	8.62	1.00
Uzuri	Hodenhagen	69.62	17.05	165.11	53.72	5.33	3.69	6.89	1.18
Kianga	Hodenhagen	56.05	16.02	146.23	43.78	5.16	4.04	7.41	1.18
Claudia	Hodenhagen	58.73	16.50	123.90	48.38	6.12	4.89	8.29	1.25
Doris	Hodenhagen	20.86	2.94	56.93	14.15	9.50	6.80	16.86	2.43
Cera	Gelsenkirchen	12.25	8.47	17.61	2.40	4.97	3.20	7.67	1.19
Tamu	Gelsenkirchen	12.45	7.60	17.58	3.17	5.79	1.89	9.83	2.44
Clara	Schwerin	55.05	17.76	102.72	33.47	11.10	6.29	20.89	4.07
Karen	Schwerin	46.88	18.19	96.3	22.71	11.20	8.09	15.71	2.01
Jane	Münster	37.25	10.32	91.64	28.62	6.77	3.41	12.43	1.99
Vicky	Münster	12.41	9.35	14.91	1.79	8.94	6.83	11.36	1.79
Yoruba	Amnéville	20.87	9.35	47.93	9.14	15.14	9.78	20.89	3.41
Hekaw	Amnéville	17.79	7.85	44.7	10.09	13.12	7.22	19.34	2.72
Lucy *	Amnéville	389.05	175.35	791.4	222.60	17.74	12.62	21.92	2.74
Tala	Amnéville	43.21	6.24	161.1	46.13	11.35	5.16	15.74	2.34
Jamala	Knuthenborg	48.39	7.02	105.18	29.22	-	-	-	-
Bodil	Knuthenborg	35.41	4.65	270.54	48.49	-	-	-	-
Sana	Planète Sauvage	7.36	6.0	8.73	1.11	10.27	6.83	13.02	3.02

* Study female was pregnant during observation period

5

General Discussion

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Chapter 5

General Discussion

The bedrocks of modern zoos are known to comprise not only entertainment but also public education and research. In addition, zoos play a substantial role in *ex situ* conservation, which facilitates the strengthening of viable populations and the preservation of genetic diversity by supporting *in situ* conservation measures yet also by breeding threatened or rare species in captivity (OWEN *et al.* 2004). Thereby, successful breeding requires a steady reproduction rate, which can only be ensured with adequate monitoring. With regards to their complexity, practical implementation and accuracy, the most common monitoring strategies (behavioural, endocrine and bioacoustic) all have their advantages and disadvantages. The key to suitable reproductive monitoring is the integration of all three of these strategies in order to provide comprehensive and multidimensional insights into the behaviour and physiology of the animals and thus, establish definitive indicators that take findings from all three monitoring measures into account (WHITHAM & WIELEBNOWSKI 2013).

Such elaborate monitoring is of particular importance for poorly breeding species, such as the white rhinoceros, which has been subject of interest in numerous studies that have been able to identify multifactorial causes for the impaired reproductive rates in captivity. The findings indicated a variety of factors potentially compromising captive breeding in white rhinoceroses, ranging from endocrinological and generative factors in both sexes to social components. However, so far, the different causes have not been systematically or directly linked with each other.

Consequently, the present work aimed to combine techniques from three monitoring strategies and analyse the collected data in the common framework of social structure and reproductive state found in captive white rhinoceroses. In order to do so, behavioural observations were simultaneously complemented with acoustic recordings, while the endocrine state of the animals during the observation period was determined using faecal samples.

In the following sections, the identification of social relationships based on vocal networks (*Chapter 2*), the effect of females' receptivity on the androgen levels, behaviour and vocalisation in males (*Chapter 3*), and the concurrence of endocrine levels and behaviour as well as vocalisation in receptive females (*Chapter 4*) are discussed with regard to potential implementations for reproductive monitoring in captive Southern white rhinoceroses.

5. 1 | Identifying social relationships based on vocal networks

While generating social networks that reflect the structure and relationships in a group based on interactions or spatial measurements between group members is a common practice in socio-biological research, the transfer of these methods to vocal communication network analyses has not been applied to the same extent yet. Nevertheless, the potential of vocal networks, a diagram visualising sender and receiver of vocalisations as connected nodes, to not only reflect directionality and frequency of the vocal exchanges, but also the social structure within a group, could be described in several animal species (e.g., FEDUREK *et al.* 2013, KULAHCI *et al.* 2015, LEMASSON *et al.* 2014, MITANI 1986). Applying the same indicators for vocal interactions in white rhinoceroses poses a difficulty, as this species has rather loose social associations, and low levels of responsiveness during interactions. As such, the detection of sender-receiver dyads is impeded.

In the study presented in *Chapter 2* these issues have been resolved by implementing a proximity-based approach, which considers all neighbouring group members at a distance of ≤ 1 adult body length to the sender as potential receivers. This approach enabled clearly structured vocal networks of the four most common call types (Hiss, Grunt, Pant, Snort: *Figure 2.1*) in seven different Southern white rhinoceros groups (*Figure 2.2.*, *Figure 2.5*) to be established. The significant sex-specific differences in call rates identified for the aggressive call types Hiss and Grunt, and the cohesive contact call Pant, imply contrasting social roles for females and males. While females act more dominant and adverse, especially towards males (*Figure 2.4*), and utter aggressive calls at significantly higher rates, males appear rather defensive despite also advertising, as indicated by the significantly higher Pant call rates.

This sex-specific call usage and the respective behavioural functions found in captivity correspond to what has also been described in the wild. Females often react in a rejecting manner towards dominant males – responding with threatening aggressive calls, especially when accompanied by their calf – while males incessantly investigate the females they encounter in their territories (OWEN-SMITH 1975, PENNY 1987, PIENAAR 1994). However, several discrepancies can still be found between captive and wild conditions, especially regarding vocalisations.

The Pant call turned out to be of particular interest. Despite the fact that this call type is often mentioned in the context of male courtship and mating behaviour (POLICHT *et al.* 2008), it is clearly not exclusive to a particular sex or age category. Adult females as well as juveniles utter Pants as well, though in different contexts. Whereas the majority of the adult males pant when they are approaching females, adult females generally utter Pants in same-sex contexts,

while juveniles emit the call type when in state of high excitement if close to their mothers or peers (LINN *et al.* 2018). These findings suggest that different functions of the Pant call may exist, namely as a mating call for males and a socio-positive cohesive call for females and juveniles.

This differentiation falls in line with a variation of the Pant call, known as Hic, which has been described as being particularly characteristic for wild white rhinoceros bulls during courtship (CINKOVÁ & POLICHT 2016, CINKOVÁ & SHRADER 2020, OWEN-SMITH 1973). According to Cinková and Shrader (2020) the Hic call contains fewer inhalation than exhalation elements and a higher proportion of short inhalation elements compared to the Pant call. However, in the present study, only two bulls were identified as having uttered calls that would match this description (*personal observation*). In addition, the study by Policht *et al.* (2008), which first characterised the vocal repertoire in white rhinoceroses in captivity using multiparametric acoustic analysis, could not find significant differences between a male Hic and a female Pant.

Viewed together, these findings suggest that the differences between the two call variations Pant and Hic might be more pronounced in the wild, where the males are able to establish and maintain territories while guarding receptive females. Hence, there might be a need for two different call types in the wild but to a much lesser extent in captivity. In addition, as both call variations are characterised as repetitive sequences of inhalations and exhalations, it is likely that there is a strong relation between the call variation and the state of arousal (CINKOVÁ & POLICHT 2016). In the wild, males may be subject to different conditions and circumstances that lead to varying levels of arousal and thus, differing call variations. This would explain the apparent lack of call variations found in captivity, due to the predictable conditions.

Furthermore, while the present study statistically identified sex-specific differences in usage of call types that are substantial in the mating context, personal observations additionally revealed a call type that was exclusive to adult bulls – the so-called Squeal. This high-pitched, wailing call has been described in the wild before (OWEN-SMITH 1973, PENNY 1987, PIENAAR 1994) and was recorded in one of the study males during mating efforts. This classification falls in line with previous literature, which described the call type in the context of the bull displaying courtship while trying to prevent the female from leaving his territory (OWEN-SMITH 1973, PENNY 1987). The reason for the specific call type being so rare in captivity may be the lacking need to lure the females back, as they are prevented from leaving the area anyway. These observations suggest that sex-specific call types, which have also been described in other

mammalian species (e.g., BOUCHET *et al.* 2012, BOUCHET *et al.* 2010, BOWYER & KITCHEN 1987, FERNANDEZ-VARGAS & JOHNSTON 2015, GREEN 1981), do exist in the white rhinoceros as well. However, as the function of some of the call types may no longer be required, their expression declines considerably in captive conditions.

Overall, the present findings show that the analysis of the vocal communication in Southern white rhinoceroses provides valuable insights into group structure and social dynamics. Thereby, females acted significantly less aggressive towards each other than towards adult males, hence tolerating same-sex company more. This relationship suggests that the so-called “buddy system”, which has been described as a formation of social bonds involving subadult individuals of both sexes and adult females in the wild (SHRADER & OWEN-SMITH 2002), is also possible in captivity. Indeed, several studies conducted in zoos described a similar kind of companionship between adult females, based on food sharing and the rate of affiliative or aggressive interactions (CINKOVÁ & BIČÍK 2013, KUNEŠ & BIČÍK 2002, SWAISGOOD *et al.* 2006). During personal observations of larger groups (e.g., N=11 in *Serengeti Park Hodenhagen, Germany*) additionally social bonds between subadults and older females were detected, which corresponds even more to the natural conditions in the wild.

Last but not least, as the contact call Pant and the aggressive calls Hiss and Grunt turned out to be the most distinctive call types with regard to their occurrence and function, they could be further utilised to monitor changes within a group after the introduction of new individuals or the removal of others. For instance, persistently aggressive calls between females, or from males to females may indicate social incapability, which could lead to enhanced stress levels that would affect the overall well-being and ultimately the reproductive capacity, as suggested by Carlstead and Brown (2005) and Metrione *et al.* (2007). Furthermore, the total absence of Pant calls in males may indicate a lack of interest in the available females and, by implication, point towards reproductive impairments on a physiological level.

5. 2 | How male endocrine state, behaviour and vocalisation are affected by female receptivity

In a variety of mammalian taxa it has been repeatedly shown that androgen levels in males are not only linked to the display of socio-sexual behaviour, such as intra-sexual aggression and courtship (e.g., GANSWINDT *et al.* 2005, GOULD & ZIEGLER 2007, LINCOLN *et al.* 1972), but also to a variety of bioacoustic parameters such as modified call structure, increased call rate and specific call usage (e.g., CHARLTON *et al.* 2011, GALEOTTI *et al.* 1997, HIGHAM *et al.* 2013, PASCH *et al.* 2011, ZIMMERMANN 1996). In some species, especially those

that are seasonal breeders, the concurrence of peaks in male testosterone levels – hence, their display of sexual behaviour – and the female oestrus is essential, as only the precise synchronisation of the physiological and behavioural reproductive states of both sexes enables successful mating (e.g., BONNEY *et al.* 1982, KLEIMAN 1983, YAGIL & ETZION 1980). However, in Southern white rhinoceros males, no such concurrence has been investigated systematically until now. Furthermore, there are only inconsistent findings on how male androgen levels are linked to the females' receptive phase, and how they might be reflected in behavioural and vocal parameters. In order to help bridge this gap, the study presented in *Chapter 3* investigated the endocrine, behavioural and vocal indicators of female receptivity in male Southern white rhinoceroses.

First and foremost, the results of the study support the previous findings of testosterone levels increasing with sexual maturation (KRETZSCHMAR *et al.* 2004, VERVERS 2018) (*Figure 3.1*), thereby emphasising the importance of androgens for the development of secondary male characteristics that emerge with the onset of puberty. The isolated case of a study male that was sampled as a two-year-old juvenile in *Serengeti Park Hodenhagen* and four years later as a sexually mature male in *Planète Sauvage*, additionally confirms the increase in androgen metabolite levels in white rhinoceros males throughout adolescence.

Furthermore, it was possible to detect evidence of declining androgen metabolite levels in older males, which falls in line with reports from other mammalian species that have described a drop in androgen concentration in advanced age (e.g., ALTMANN *et al.* 2010, MACHIDA *et al.* 1981, THOMPSON *et al.* 2012). Despite providing initial insights into the ontogenetic development of androgen concentrations in male Southern white rhinoceroses – and although certainly of interest – these findings do not allow any further statement to be made regarding if and how the decreased androgen concentrations might affect the males' fertility. Even though the process of spermatogenesis is generally known to be highly testosterone-dependent (PRESTON *et al.* 2012), further studies including larger sample sizes and analyses of physiological parameters, such as sperm production and quality, would be necessary for reliable conclusions to be drawn. Nevertheless, since one of the oldest study males clearly displayed sexual behaviour towards receptive females, it seems that decreasing androgen levels do not conclusively affect the reproductive motivation of the males.

Upon further examination, a potential effect of previous breeding on the faecal testosterone metabolite levels could be ruled out, as there was no evidence of an impact resulting from sexually mature males having successfully sired offspring before or not. This outcome matches the findings from studies both in captivity, where no differences were found

between proven and non-proven bulls (BROWN *et al.* 2001), as well as in the wild, where there was also no link found between androgen levels and reproductive success (KRETZSCHMAR *et al.* 2020). Withal, a clear effect of female receptivity on fTM levels was revealed - with males that had direct contact with receptive females having higher androgen metabolite levels than males that did not have any contact with receptive females. Thus, the female effect that has already been described in several species such as horses (MCDONNELL & MURRAY 1995), hyenas (DLONIAK *et al.* 2006), rhesus macaques (ROSE *et al.* 1972) and chimpanzees (MULLER & WRANGHAM 2004) could be confirmed with regard to the Southern white rhinoceros. This outcome is also consistent with the results of previous studies by Christensen *et al.* (2009) and Kretzschmar *et al.* (2004), who also both found higher androgen levels in white rhinoceros males when they were accompanied by females, compared to when they were alone.

Despite the evident link between the elevated androgen levels in males and their contact with receptive females, future studies that explore the causality of this interrelation are necessary. Endocrine monitoring before and after a transfer to a new group, as well as comparisons with single-housed adult males would provide further information on the factors and mechanisms affecting androgen concentrations in white rhinoceros males. Indications from reports on individual cases exist, in which white rhinoceros males have successfully bred again after being transferred to new groups, despite years of reproductive inactivity (SCHWARZENBERGER *et al.* 1999). However, a direct link to their testosterone levels has not yet been investigated.

Interestingly, the fTM levels of the males that had access to receptive females remained unchanged throughout the females' cycles, apparently independent of receptive or non-receptive periods. In contrast, male behavioural and vocal patterns varied significantly between the receptive and non-receptive periods (*Figure 3.2*). During the females' receptive period, males demonstrably spent more time in close proximity to the females, displayed affiliative interactions such as *body* and *snout contact* as well as *following*, at a significantly higher rate. Additionally, the males uttered Pant and Hiss calls almost exclusively during this period. This discrepancy, throughout the different stages of the female cycle, between male androgen levels on the one hand and their behavioural and vocal profiles on the other hand suggests that testosterone might not be the proximate driver of the behavioural and vocal changes.

In view of potential propositions for breeding Southern white rhinoceroses in captivity, the findings of the study provided some valuable insights. For instance, as there is no evidence for an effect of previous reproductive success but of female's receptivity on the androgen levels, a transfer of a formerly soundly breeding bull to a new group might not guarantee a continued

breeding success, as it greatly relies on the females' reproductive capacity. Even though Patton *et al.* (1999) and Schwarzenberger *et al.* (2003) reported cases that indicate the possibility of a new mating partner eliciting an oestrous in acyclic females, to date there are no systematic investigations available on the extent of the impact new mating partners might have on reproduction. Therefore, there is no information available whether proven or non-proven males or the ones with low or high androgen levels would make a difference for reproductive success in white rhinoceroses. As witnessed in horses - a phylogenetically close relative to the *Rhinocerotidae* family - the presence of a stallion indeed affects the cyclicity of mares (e.g., SINCLAIR *et al.* 2020, WESPI *et al.* 2014). Given this fact, this kind of male effect might also be assumed for white rhinoceroses. Indeed, a field study by van der Goot *et al.* (2015) reported on a case of a females that stopped cycling after the removal of the territorial bull and started again when a new mating partner was introduced.

Overall, it remains to be noted that an established reproductive capacity of one of the mating partners does not ensure reproductive success of the breeding pair, as both sides must be examined first.

5. 3 | How female endocrine state, behaviour and vocalisation change throughout oestrous

A number of previous studies investigated the hormone profiles of white rhinoceros females in captivity and repeatedly described irregular and aberrant cycles, as well as prolonged luteal phases that might cause pathologies of the female reproductive tract (BROWN *et al.* 2001, HERMES *et al.* 2004, HERMES *et al.* 2006, PATTON *et al.* 1999, SCHWARZENBERGER *et al.* 1998). However, none of these studies has yet created a systematic link between the mentioned endocrine findings and behaviour or vocalisation. Even though some studies mentioned a coincidence of ovulation, which has been identified based on nadirs in progesterone concentrations, and mating occurrence (BROWN *et al.* 2001, PATTON *et al.* 1999, RADCLIFFE *et al.* 1997, SCHWARZENBERGER *et al.* 1998, VAN DER GOOT *et al.* 2015), these references were based on random behavioural observations rather than on continuous data collection and systematic monitoring. Moreover, neither olfactory nor vocal behaviour have been specifically included in these studies at all. With the aim of linking these interdisciplinary aspects, the study presented in *Chapter 4* provided insights into the changes of behaviour and vocalisation as well as of the endocrine profile in Southern white rhinoceros females throughout their oestrous cycle.

Eight out of 21 study females that were kept in direct contact with adult bulls also accepted sexual interactions (*head placing, mounting* and ultimately *copulation*) from them

during the observation period, thereby signalling their receptivity (BEACH 1976). During this phase, several behavioural patterns distinctly peaked compared to the non-receptive periods before and after (*Figure 4.1*): The females spent significantly more time in close proximity to the bulls and directed more affiliative interactions towards them, particularly the *presenting* behaviour of lifting up the tail. Moreover, females displayed the *marking* behaviour of intermittently spraying their urine exclusively during the receptive period. Changes in vocal behaviour were not particularly pronounced during receptivity. However, the very few females that uttered Pants towards males, uttered them exclusively during their receptive period.

For five of these eight females that signalled their receptivity with distinct behavioural and vocal indicators, the study identified a hormonal oestrus based on their profiles of faecal progesterone metabolite concentrations (*Figure 4.2 A*). A distinct cyclic development of progesterone metabolite levels enabled the discrimination between the follicular phase characterised by fPM levels below baseline, and the luteal phase with increased fPM levels at maximum level. Thereby, the follicular phase, and thus the nadir of fPM concentrations, coincided with the receptive period, highlighting the behavioural patterns and call rates that peaked during this phase as reliable indicators of female receptivity.

With regards to faecal oestrogen metabolites no cyclic development was detected that could have identified the hormonal oestrus. This limitation has been previously noted in several studies that described faecal oestrogen metabolites as a non-suitable indicator for ovarian activity in white rhinoceros females (e.g., BROWN *et al.* 2001, ROTH *et al.* 2018). It appears this assessment could not be improved by means of a higher sampling rate either, as even in study females that were sampled daily, the fEM levels still did not show any cyclic patterns. This would match the explanation of Schwarzenberger and Brown (2013), who stated that the follicles of white rhinoceros females generally produce too little oestrogen to reliably indicate follicular development based on faecal or urinary samples. Accordingly, also in the present study no peak could be detected in the fEM concentrations that would have coincided with the receptive period.

From two of the eight females that displayed behavioural and vocal indicators of their receptivity, an insufficient number of samples was collected during the observation period, impeding the determination of a hormonal oestrus. However, when comparing their hormone parameters with those of the other study females, it may be concluded that they very likely also had an oestrus during the observation period, since both had fPM values that corresponded more to the increased values of the females with an oestrous cycle rather than to the ones without.

Hence, the percentage of coincidence between the receptive period signalled by behavioural and vocal indicators and the hormonal oestrus could be expanded almost to the fullest.

However, one exceptional case of a study female that displayed sexual behaviour, yet did not have a corresponding hormonal profile (*Figure 4.2 B*), demonstrated how important it is to acknowledge individual deviations from the norm, in order to include all potential contingencies in reproductive monitoring. The female in question did accept the male mating efforts, but not on several consecutive days as seen in the other study females. Instead, the accepted sexual interactions that defined the female's receptive period were observed on only very few occasions with several days in between. This divergence from what has been described in other cases matched the endocrine profile, as no hormonal oestrus could be detected. Thus, this particular female apparently experienced an abnormal cycle, demonstrating that aberrant hormone profiles can also be reflected by respective divergent behavioural patterns.

Some further noteworthy exceptional cases arose from study females that did not signal their receptivity. One of them had direct contact with an adult male and according to her fPM profile also had an oestrus during the observation period (*Figure 4.3 B*). However, this study female persistently rejected the bull whenever he was approaching her and did not display either *presenting* or *marking* behaviour. In view of the fact that the two other females in the group both had an oestrus as well, while they were distinctly signalling their receptivity to the bull and ultimately copulating, the bull's reproductive inability can be ruled out. A possible reason for the female's lack of sexual behaviour, however, could be her age, suggesting that old white rhinoceros females might display an endocrine cycle but not the respective behaviour. In fact, according to the European Studbook of white rhinoceroses, the effective reproductive age of females ends with 36 years (VERSTEEGE 2020). The study female in question was 43 years old during the observation period (*Table 4.1*) and hence, not considered reproductively active anymore. The general characterisation of the reproductive lifespan of females in mammalian species describes a dysregulation of the pituitary-hypothalamic-ovarian axis, leading to a decline in ovarian hormonal levels with increasing age (reviewed by LEMAÎTRE & GAILLARD 2017). Hence, the state of anaphrodisia, also known as the "silent heat", detected in the study female could be a result of gonadotropin deficiency caused by her advanced age (GRUNERT *et al.* 1996). However, further investigations including the effects of disease, diet or detrimental housing conditions would be needed in order to identify the cause for the unnoticed oestrus.

Finally, the example of the study female that had a hormonal oestrus but no direct contact with an adult bull (*Figure 4.3 C*), further supports the previous characterisation of white rhinoceros females as being spontaneous ovulators (ROTH 2006, SCHWARZENBERGER & BROWN

2013). However, in contrast to the majority of the other study females that proved to have had an oestrus as well, this female was not observed to show any of the behavioural and vocal indicators such as *presenting*, *marking* and *panting*. This suggests that in order to display behaviour that would appropriately signal their receptivity, white rhinoceros females indeed need a male stimulus.

5.4 | Indicators of receptivity in males and females

When considering the findings of the presented studies in the common context of social structures and the reproduction of white rhinoceroses in captivity, distinct behavioural patterns are revealed for both sexes. Thereby, initiating sexual interactions such as *head placing*, *mounting* and *copulation* on the part of the males, and accepting those on the part of the females coincided with the hormone oestrus of the females and thus, clearly identified their receptive period (*Figure 5.1 A+B*).



Figure 5.1: Behavioural indicators of female receptivity in Southern white rhinoceroses. **A)** Increased close proximity and body contact between male and female. **B)** Male places his head on the female's rear. **C)** Male mounts the female with erect penis followed by copulation. **D)** Ano-genital sniffing of female rear by the male. **E)** Female is presenting her vulva by lifting up her tail while being followed by the male. **F)** Female intermittently sprays her urine (circled area) with the male close by.

In addition, further indicators of female receptivity could be determined for both, males as well as females. Both sexes spent significantly more time in close proximity to each other and exchanged more affiliative interactions such as *body* and *snout contact* during the females' receptive period (*Figure 5.1 C+D*). However, on average, the bulls displayed twice as much affiliative behaviour towards the females than the other way round (*Figure 3.2, Figure 4.1*). Furthermore, particularly for males, the call rates of Pants and Hisses were conspicuous, as both

call types were uttered almost exclusively during the females' oestrus. In contrast, it was the olfactory behaviour that stood out most in females, as *marking* behaviour distinctly peaked during oestrus (*Figure 5.1 F*). Moreover, females only displayed *presenting* behaviour during their receptive period (*Figure 5.1 E*).

Consequently, males and females seem to have different characteristics of behavioural changes in the reproductive context (*Table 5.1*). While males primarily seem to indicate their interest and desire for mating via vocal communication, females seem to signal their readiness for mating via olfactory communication by marking or lifting up their tail, and in doing so, provide the opportunity for the males to absorb essential olfactory cues.

Table 5.1: Overview of the sex-specific differences in behaviour and vocalisation in the Southern white rhinoceros.

	Males	Females
Most common call type	Pant	Hiss
Most common potential receiver	adult females	adult males
Most common indicators of female receptivity	<ul style="list-style-type: none"> ● increase of time spent in close proximity to female ● affiliative behaviour <ul style="list-style-type: none"> - <i>approaching</i> - <i>following</i> - <i>body/snout contact</i> - <i>naso-nasal/ano-genital sniffing</i> ● sexual behaviour <ul style="list-style-type: none"> - <i>head placing</i> - <i>mounting</i> - <i>copulation</i> ● increase of vocal activity, especially Pant call rate 	<ul style="list-style-type: none"> ● accepting affiliative and sexual behaviour from bull ● increase of time spent in close proximity to bull ● increase of affiliative interactions towards bull <ul style="list-style-type: none"> - <i>presenting</i> ● increase of olfactory behaviour <ul style="list-style-type: none"> - <i>marking</i>

Previous studies on the importance of olfactory communication in white rhinoceroses describe how males absorb volatile organic compounds from the females' urine and dung, and react with increased interest and repeated visits of the dung piles when oestrous odour profiles were applied (MARNEWECK *et al.* 2017). Therefore, the change in the chemical composition of female olfactory cues during oestrus, which the males absorb during *sniffing* and *flehming*, may act as a crucial trigger for the males, eventually leading to the respective behavioural and vocal adjustments. As the rates of male olfactory behaviour remained unaffected throughout the female cycle in the present study, it might be assumed that it is not necessarily the quantity of olfactory information that is significant for the changes in behaviour and vocalisation, but rather the quality.

These sex-specific differences reflect the social structure of the species in the wild, where the larger territories of the females overlap with smaller territories of several males (PENNY 1987, PIENAAR 1994, SHRADER & OWEN-SMITH 2002). As white rhinoceroses are

known to use communal dung piles in order to exchange information about each other (CINKOVÁ & POLICHT 2015, MARNEWECK *et al.* 2018), it can be assumed that females in oestrus are leaving an augmented amount of olfactory cues, in order to increase the chances for neighbouring bulls to perceive their reproductive state. Eventually, the bulls react to these signals by finding the respective female, approaching her while panting and courting her, for up to several weeks, in order to ensure that no other male gets the chance to mate (OWEN-SMITH 1975, PIENAAR 1994). This natural behaviour appears to not have changed in captive conditions.

5. 5 | Implementations for reproductive monitoring in captivity

According to the present studies, the female' receptivity in captive conditions usually lasts from one to five days. During this period the behavioural and vocal indicators in males and females can be observed on an almost daily basis. Ergo, erratic and sporadic occurrence of the indicators – especially in females – could be a sign of an impairment in ovarian activity, in some form or another, and hence, serve as a reason to examine the respective female further.

The evident importance of olfactory communication with regard to reproduction as presented in the study, emphasises the necessity for the males to regularly absorb female olfactory cues. Hence, facilities that keep males and females separated, should at least provide the males intermittent access to the females' pen or enclosure. Subsequent Pant calls and restless behaviour on behalf of the male – while he is trying to find and approach the female – would therefore be reliable signs, calling to unite the breeding pair. In addition, it might be assumed that males that do not receive any or only scarce olfactory cues from females might not adjust their behaviour accordingly. A complete separation of males and females should therefore be avoided, as successful breeding requires preceding opportunities for olfactory as well as vocal exchange between the sexes.

Beyond that, vocal communication has proven to be relevant for the reproduction in white rhinoceroses as well. However, the present findings revealed some difficulties of applying bioacoustic reproductive monitoring in this species. The calls that featured sex-specific differences and turned out to be essential in the mating context, namely Hiss and especially Pant, are both noisy call types that do not exhibit harmonic structures (POLICHT *et al.* 2008), which complicates the analysis of potential acoustic correlates of e.g., oestrus state. Besides, the background noise of the environment inhibits proper detection. Although previous studies have shown that especially the Pant calls entail individual signatures and provide information on the sex, species and motivation to the receiver (CINKOVÁ & POLICHT 2014, CINKOVÁ & POLICHT 2016, CINKOVÁ & SHRADER 2020), these correlates have only been described for male

white rhinoceroses, and required a complex data analysis that would not be suitable for the daily life praxis at a zoo.

Instead, what might be feasible is to focus the monitoring on the overall occurrence of male Pant calls, as they appear to be confined to the females' receptive phase. An automated detector that could merely recognise a call as a Pant, thus recording its presence or absence, would be quite conceivable and certainly beneficial for reproductive monitoring in white rhinoceroses. Similar detectors have already been used in livestock studies and were able to reveal some conclusive information on individual oestrus vocalisation in cattle to the researchers (RÖTTGEN *et al.* 2020). Hence, the development of an automatic call-recognition system for white rhinoceroses appears to be a promising approach that should be explored in further studies.

Adjusting husbandry conditions to mimic natural social organisation by creating opportunities for males and females to part ways using visual and spatial barriers in the enclosure and periodically separating males from the group might furthermore promote reproduction. The studies presented in this thesis show that females generally behave in a rather rejecting manner towards males and, except for the receptive period, actively keep them at a distance using aggressive call types such as Hiss and Grunt, which are often accompanied by behaviours such as *displacing* or *attacking*. Therefore, housing the whole group together at all times might either lead to increased stress or, quite the contrary, unintentional habituation that would result in behavioural changes crucial for mating, in that they would not be displayed intensively enough to attract the mating partner (LINDBURG & FITCH-SNYDER 1994). This kind of sibling-like relationship has been especially observed in white rhinoceroses that were kept solely as a breeding pair, which often led to a silent oestrus in the female (SCHWARZENBERGER *et al.* 1999). Conclusively, simulating natural conditions by temporarily separating the adult bulls from the group, creating regular possibilities for the breeding pair to exchange olfactory cues and then subsequently reuniting the breeding pair during the females' receptive periods would be a worthwhile approach beneficial to the reproduction of white rhinoceroses.

Finally, by combining behavioural, endocrine and bioacoustic measures, this thesis has established behavioural and vocal indicators that create an intuitive and substantial basis for the reproductive monitoring of captive Southern white rhinoceroses, on a daily basis. Individual alterations of these indicators might give reason for a further examination of the respective animal in order to determine the underlying causes. In these cases, complementary monitoring tools, such as further endocrine analyses or ultrasonic tests, would provide in-depth information pertaining to the roots of the anomalies (e.g., HERMES *et al.* 2006, RADCLIFFE *et al.* 1997). In

addition, the thesis has also outlined potential approaches on how to counteract the lack of the behavioural and vocal indicators mentioned, whereby the main focus is on the exchange of acoustic and olfactory stimuli between the sexes. Hence, since olfactory stimuli are evidently crucial for the display of sexual behaviour in white rhinoceroses, follow-up studies could investigate whether the exchange of hay soaked in the scent of oestrous females between zoos elicits respective behavioural changes in the males.

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Acknowledgements

This thesis is the outcome of the effort and support of many people to whom I am extremely grateful.

First and foremost, I would like to thank my first supervisor, **Dr Marina Scheumann**, who offered me the chance to work on this incredible topic. I could not have asked for a better mentor. You always had my back, you motivated me, challenged me and gave me all the opportunities to develop further, become independent and work beyond my imposter syndrome. I was very lucky to have had a doctoral supervisor like you and I will for ever be grateful.

I would like to offer my sincere gratitude to **Prof Dr Ursula Siebert** and **Dr Angela Stöger-Horwath** for agreeing to join my supervision group and for encouraging and supporting me with invaluable expertise and insightful feedback that broadened my view and sharpened my thinking.

I am also profoundly grateful for the support of **Dr Jella Wauters**, who never got tired of my questions on the chances and pitfalls of endocrinology. I learned so much from our collaboration and it's been a great pleasure working with you, I hope this is not the end of it. *Heel erg bedankt!*

At this point, I would also like to express many many thanks to **Mareen Albrecht** and **Katrin Paschmionka** at IZW Berlin for their technical assistance for the endocrine monitoring.

In addition, I would like to thank my co-authors for their substantial contributions. **Brenda Chaignon**, I very much appreciated your hard work and your endless motivation as well as your delightful company at my very first study site. **Sabrina Linn**, thank you so much for paving the way for this incredible project and providing me with valuable insider information and your know-how. **Dr Martin Dehnhard**, thank you for offering your expertise and sharing your scientific experience.

Furthermore, I would like to offer my special thanks to the curators and zookeepers all around Europe – this project would have never been possible without your help and advice. To **Sabrina Höft**, **Jochen Lengger**, **Dr Dirk Wewers**, **David Bilger**, **Rasmus Nielsen**, **Martin Boye** and so many more - thank you for introducing me to the zoo community and allowing me being a part of it. I hope we will meet again one time or another!

I would also like to extend my sincere thanks to **Vera Pfannerstill**, my fellow rhino enthusiast. I am very happy that this project brought us together and gave us the chance to be more than just colleagues. I appreciated your company and learned a lot from your persistence and patience. I hope our ways will continue crossing over and over again in the future.

I would also like to thank all my wonderful colleagues at the **Institute of Zoology** for making me feel welcome right away, entertaining me during lunch breaks and preparing me for the scientific world with your constructive feedback and support.

I could not have imagined enjoying working at the Institute of Zoology as much as I did without my partners in crime – **Dr Annette Klein** and **Sönke von den Berg**.

Annette, I truly have no words for how incredibly lucky I am to have found a friend like you. The last three years would have been miserable without your contagious nature of always trying higher, better, faster. Thank you for always reminding me how much more there is to see and to do in this world! Sönke, thank you for being your matchless self. Getting coffee, exchanging some sarcasm par excellence, looking forward to Friday's special and simply knowing whom to call when the technology is striking again became an irreplaceable routine for me.

Undoubtedly, I could not have got through my doctorate without having my loving family by my side, whom I would like to express my deepest gratitude.

Dr Eduard Jenikejew, *батько*, you have always been the biggest inspiration to me on what you can achieve with hard work and dedication and despite not always agreeing on everything, I always cherished your advice and opinion. **Tatjana Fedschenko**, *mami*, thank you for giving me the space and strength for growing into the person I am today and always sticking up for me. **Vera Jenikejew**, my little sister, thank you for always reminding me how to be your truest self and letting me watch you grow into this amazing, confident person that you are today. *Я вас дуже люблю!*

Last but not least, my dearest friends – **Camilla Cenni**, **Dr Falk Eckhardt**, **Antonia Ewald**, **Daniel Ewers**, **Florian Fiß**, **Alina Glaub**, **Dr Lea Hembach**, **Theresa Höfling**, **Moritz Jäger**, **Nico Klasen**, **Karl Niklas Martin**, **Jennifer Pede**, **Max Radke**, **Dr Bako Rasolofoniaina**, **Dr Susanne Sangenstedt**, **Lea Steinbach**, **Dr Melina Vallbracht** and **Genta Zhuta**. Thank you all for being part of my life for so many years now. To many more to come!

Finally, this 3-year journey would not have been possible without the financial support of **Deutsche Forschungsgemeinschaft**, to whom I am sincerely grateful.

My last "Thank you" goes out to my precious **rhinos**. What a ride! I hope we will all make it...