

BREEDING ON THE BRINK OF EXTINCTION

WHAT CAN WE LEARN FROM GAME-RANCHED WHITE RHINOCEROSES (*CERATOTHERIUM SIMUM SIMUM*)?

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WHITE RHINOCEROSES (*CERATOTHERIUM SIMUM SIMUM*)?

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Cover photo: Image of 'Hope', the rhinoceros cow that initially survived a poaching attack and received multiple treatments from the 'Saving The Survivors'-team. Hope became a symbol for all surviving victims of poaching incidents (Adrian Steirn, www.adriansteirn.com).

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
ALH	Amplitude of Lateral Head Displacement
ART	Artificial Reproductive Technologies
BC	Berliner Cryomedium
BCF	Beat Cross Frequency
CASA	Computer Assisted Sperm Analysis
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CR	Calving Rate
DMSO	Dimethyl Sulfoxide
DW	Dry Weight
eCG	Equine Chorionic Gonadotropin
EED	Early Embryonic Death
EIA	Enzyme Immunoassay
ET	Embryo Transfer
fAM	Faecal Androgen Metabolites
fPM	Faecal Progesterone Metabolites
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
ICI	Inter-Calving Interval
ICSI	Intracytoplasmic sperm injection
IQR	Interquartile Range
IVF	In vitro Fertilisation
LH	Luteinising Hormone
LIN	Linearity ($VSL/VCL \times 100\%$)
LSM	Least Square Means
NWR	Northern White Rhinoceros
OPU	Ovum Pick-Up
SADC	South African Development Community
STR	Straightness ($VAP/VCL \times 100\%$)
SWR	Southern White Rhinoceros
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight Line Velocity

CHAPTER 1

GENERAL INTRODUCTION

Modified from:

Ververs C., van Zijll Langhout M., Govaere J., Van Soom A. (2015). Features of reproduction and assisted reproduction in the white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros. Vlaams Diergeneeskundig tijdschrift 2015; 84: 175-186.

1.1 *Ceratotherium simum* spp from near threatened to

The African white rhinoceros is one of the keystone species in Southern Africa (Owen-Smith 1987). It has been proposed that the white rhino acts as an influential ecosystem engineer, creating and maintaining short grass swards, which alters the habitat for other grazers in ecosystem management. Owen-Smith (1987) coined the term megaherbivores for very large herbivores with an adult bodyweight over 1000 kg. He noted that large body size renders these herbivores largely immune to (non-human) predation, while their bulk feeding allows them to digest food of a lower quality than that required by smaller herbivores. He suggested that megaherbivores would therefore be less affected by predation or environmental fluctuations (for example, drought) than mesoherbivores (herbivores 50-500 kg) and their populations would be maintained at high density causing major impacts upon their environment. Contrary to megaherbivores such as black rhinoceros, who are mainly browsers, the white rhinoceros is a grazer (Waldram et al. 2008). Elephants are grazers and browsers, but prefer to graze depending on availability.

The socio-economic value of rhinoceroses worldwide includes several aspects. The five extant species of rhinoceroses on this planet are part of the few species left which still are closely related to prehistoric and now extinct species (Orlando et al. 2003). These animals managed to survive for millions of years, throughout earth-changing developments, and have recovered once before from a tremendous population decline in the 20th century. The southern white rhinoceros (SWR) was exemplary for a major conservation success story. With numbers as low as 50 in the wild in the early 1900s, the population of this subspecies has recently increased to almost 20,000 individuals (Emslie & Brooks 1999; Linklater 2003; Amin et al. 2006; Lindsey et al. 2009; Emslie et al. 2012; Emslie R. 2017). Sadly, due to the loss of habitat and increased poaching the survival of this species is again very much at risk. The white rhinoceros (*Ceratotherium simum*) is considered to exist of two subspecies: the southern white rhinoceros (*Ceratotherium simum simum*), and the near extinct Northern white rhinoceros (*Ceratotherium simum cottoni*). South Africa represents the major habitat for the southern white rhinoceros, conserving 16.255 individuals in the wild in 2007 (Ripple et al. 2015). However, poaching has increased dramatically since 2008, due to a huge demand for rhino horn, and is causing a rapid

decline in wild rhinoceros populations and more particularly, of southern white rhinoceros numbers in South Africa. Being a member of the so called 'Big Five' animals in Africa, including rhinoceroses, African buffalos, elephants, lions and leopards, the white rhinoceroses attract millions of tourists to Southern Africa every year. Although individual animals or small groups living in zoological institutions worldwide, observing these animals in their natural habitat, the African bush, is the highest valued and most fulfilling experience. The safari industry, with both international and national interests, is of great economic value for Southern African countries. A recent survey showed that having the 'Big Five' animals present in a reserve contributes greatly to tourist satisfaction and also willingness to pay (more) money (Lindsey et al. 2007; Maciejewski & Kerley 2014). However, opportunities to see free-roaming white rhinoceros in national parks in Southern Africa have been reduced due to the present poaching crisis. Therefore, intensive protection of these animals is required nowadays. This implies that security measures and costs rise significantly with increased poaching. Poaching as such will negatively affect economic benefits of tourism if safety matters exclude tourists from visiting national parks. When man-induced deaths equal birth rates of a certain species, no economic benefit can be made by sales of surplus animals. Investing money into maintenance, conservation and protection of these private properties and national reserves will decrease if no income can/or may be generated and long-term survival of these properties comes at risk (Rubino & Pienaar 2017).

Dwindling population numbers of wild rhinoceros are mainly related to the inherent value of horn. If the current population numbers continue to diminish at the present rate, with over 1,200 southern white rhinoceros being poached in South Africa alone in 2015 and over 1,000 in 2016, captive breeding programs may be crucial to prevent extinction. Recent endeavours to breed white rhinoceros in captivity have led to growth and expansion of the current population numbers with improved genetic diversity and an increased economic return of investment (Garnier et al. 2001). Although the southern white rhinoceros is the least threatened of the rhinoceros species, we decided to study it because it is the most approachable and still left in numbers that allow relevant research. We would like to present it as a keystone-species of which study results can benefit the other species as well.

1.1.1 Classification

The rhinoceros belongs to the Perissodactyl group or odd-toed animals, which is an order of large herbivores that may be subdivided in the suborder of Hippomorpha, to which the Equidae belong (including the horse, donkey and zebra), and a common clade, the suborder of Ceratomorpha for the families of the tapirs (Tapiridae) and rhinoceros (Rhinocerotidae). The latter consists of five extant rhinoceros species: the black rhinoceros (*Diceros bicornis*), with its four subspecies, of which the *D. b. longipes* or Western black rhinoceros is considered extinct, and the white rhinoceros (*Ceratotherium simum*), with its two subspecies (*Ceratotherium simum simum* and *Ceratotherium simum cottoni*), which live in Africa, and the Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*), which live in Asia. They differ in population numbers and level of endangerment, with some being listed as critically endangered (black, Javan, Sumatran rhinoceros) to vulnerable (Indian) and some being listed as near threatened (white) (Emslie et al. 2012). There is a contrast in conservation success between southern and northern white rhinoceros. Northern whites were once fairly numerous but are now critically endangered and near to extinction (only two left to date, as of April 2018). The southern whites however have recovered from a few individuals to a population of a few thousand nowadays, although these numbers are decreasing again due to poaching incidents in the last decades. Geneticists believe that the two sub-species split around a million years ago, with southern whites based in Southern Africa and Northern whites living in Central Africa (they once roamed in Uganda, Chad, across pre-partition Sudan, the Central African Republic (CAR) and the Democratic Republic of Congo (DRC). Skull anatomy and dental morphology by Groves *et al.* (Groves et al. 2010) clearly diagnosed the southern and northern variant of the white rhinoceros. Basic reproductive parameters in captivity have been studied in both northern and southern white rhinoceros and together with sperm morphology and social behaviour they are similar or identical for both variants (Mikulica 1991; Vahala et al. 1993; Silinski 2003). Therefore, we approach them in this study as one taxa, *Ceratotherium simum* ssp, and all our studies were performed on southern white rhinoceros (*Ceratotherium simum* ssp. *simum*).

1.1.2 *Population numbers of Ceratotherium simum ssp. simum*

With numbers as low as 50 in the wild in the early 1900s, this subspecies has recently increased to a population of almost 20.000 white rhinoceros worldwide living in the wild and another 750 white rhinoceros in captivity in zoological institutions. South Africa currently represents the major habitat for the southern white rhinoceros, conserving 16,255 individuals in the wild in 2007 (Ripple et al. 2015).

1.1.3 *Captive, semi-captive and wild*

Recent endeavours to breed white rhinoceros in captivity have led to growth and expansion of the current population numbers, with improved genetic diversity and an increased economic return of investment (Garnier et al. 2001). Most reports on reproductive performances in zoological institutions have been about individuals or small groups of rhinoceroses. Wild-caught southern white rhinoceroses will readily breed in captivity if given appropriate space and food, and if other female rhinos of breeding age are present. However, for reasons currently not understood, the reproduction rate is extremely low among captive-born southern white females (Swaisgood et al. 2006). Social hierarchy and a free choice of mating differed a lot between the different settings.

1.2 Reproductive anatomy and breeding performances of the horse and the white rhinoceros: two related species

The horse is one of the closest domestic relatives of the rhinoceros, and preferable over the donkey or zebra as a model, since reproduction in the horse has been studied at a much profounder level than in other equids. Comparing the horse model with the current reproductive data on the (white and black) rhinoceros could hence provide useful information on how to improve the breeding techniques in rhinoceros. However, it should be kept in mind that equids and rhinoceros also differ in many aspects.

1.2.1 Anatomy of the female reproductive tract

In the cycling mare (*Equus caballus*), the reproductive tract measures between 67-76 cm from the vulva to the utero-tubal junction. The ovaries are large (28 cm³), bean-shaped organs located in the sublumbar area. Ovulation of mature follicles only occurs in the ovulation fossa, because of the existence of a tough and fibrous tunica albuginea covering the external surface of the ovary. The structure of the ovary in the horse is reversed, with the cortex in the centre and the medulla (covered by the tunica albuginea) on the surface (Allen, 2005). This is unique compared to other animal species. The Fallopian tubes are long and tortuous ducts, 20 to 30 cm in length, and are divided in the infundibulum, the ampulla and the isthmus. The uterus of the mare is bicornuate, with a pronounced corpus and short horns, and the lumen is lined with prominent endometrial folds. The longitudinal folds of the thick-walled cervix are continuous with the endometrial folds. The cervix is large (5 – 7.5 cm length) and softens during oestrus. There are no cervical rings present, which enables the cervical lumen to greatly expand and contract during the oestrous cycle. The vertically oriented vulva is located ventral to the anus (Brinsko et al. 2011).

In the rhinoceros cow, the length of the reproductive tract ranges between 0.8 and 1.5 m, from the vulva to the tip of the uterine horn, depending on the species (Godfrey et al. 1991; Schaffer et al. 2001). The ovaries are very large: $34.1 \pm 4.3 \text{ cm}^3$ for white rhinoceros with regular cycles. In animals with less regular cycles, active ovaries are much smaller ($29.2 \pm 2.2 \text{ cm}^3$) and inactive ovaries are about half the size (14.7 ± 1.3^3) (Hermes et al. 2006). The ovaries are positioned within the ovarian bursa and located caudo-laterally to the kidneys (Schaffer et al. 2001). Germinal epithelium (cortex) lines the surface of the ovary, enclosing the stroma (medulla) within, similar to most domestic species. The follicles, unlike the situation in the horse, can ovulate over the entire surface (Godfrey et al. 1991). Observed inequality in the side of ovulation, although based on only a few individual animals, is more similar to the horse (Ginther & Pierson 1983; Radcliffe et al. 1997). In the rhinoceros, each Fallopian tube consists of a small tubular structure with fimbriae, not attached to the ovary at the open cranial end. The uterus is bicornuate. The anatomy of the uterus of the rhinoceros is anatomically more like the uterus of the bitch and the sow, since female rhinoceros have large uterine horns and a relatively short uterine body (Godfrey et al. 1991; Schaffer et al. 2001). The cervix, with its long and

convoluted morphology and extremely tight folds of connective tissue, may represent an obstacle for artificial insemination (AI) (Godfrey et al. 1991; Hermes et al. 2007). The presence of the hymen or hymeneal membrane at examination proves that successful mating did not happen before. This is not unique to rhinoceros, but this finding may be used as such to indicate potential reproductive problems in the white rhinoceros (Hermes et al. 2006).

1.2.1 Oestrous cycle: physiological changes and follow-up

Horses have an oestrous cycle of 22 days with 5-7 days of oestrus (Aurich 2011). They are long-day seasonal, poly-oestrous breeders. Puberty occurs around 12-18 months of age. Typically, there is no substantial post-reproductive life span. Oestrous behaviour is characterized by increased interest towards the stallion. Stallions are also attracted to a mare in heat. The mare turns her hindquarters towards the stallion and shows a typical posture with lowered pelvis and straddled hind limbs, accompanied by deviation of the tail with 'clitoral winking' (Aurich 2011). One or two major follicular waves develop per cycle. The dominant follicle reaches an average size of 40 mm at ovulation, which can even increase to a pre-ovulatory size of 55 mm, also depending on the type of breed and time during the season (Ginther et al. 2008; Aurich 2011). During oestrus, progesterone levels are low (<1 ng per mL) but they reach levels higher than 1 ng per mL three to four days after ovulation (Terblanche & Maree 1981).

After fertilization, a single spherical blastocyst (sometimes two) migrates through the uterus until day 17, the time of fixation (Ginther 1983; Leith & Ginther 1984; Ginther 1985; Allen & Stewart 2001). The placenta is of an epitheliochorial type. From day 40-42 of pregnancy, implantation starts and endometrial cups form in the placenta, which secrete a gonadotrophic hormone (eCG) (Allen 2001b). The eCG hormone supports the primary corpus luteum by its luteotropic activity and stimulates the development of accessory corpora lutea by stimulating ovarian follicles to luteinize and secrete progesterone to maintain pregnancy. Horses have a gestation period of 330-350 days (Howell & Rollins 1951; Satue et al. 2011).

For many years now, transrectal ultrasonography has been used to monitor the oestrous cycle in the mare (Ginther & Pierson 1983). It is a technique that can be

applied in the standing, non-sedated horse. Examinations can be done repeatedly over many days (Griffin & Ginther 1992). Peripheral blood progesterone concentrations can also be used to determine the stage of the cycle, due to the luteal phase being 14 days long on average. During the first 14 days of pregnancy, the progesterone levels are similar to those of the luteal phase. During the follicular phase, the level of progesterone is at baseline (Allen & Hadley 1974).

Rhinoceros are non-seasonal, poly-oestrous breeders (Garnier et al. 2002). Follicular activity starts at puberty, at the age of three to four years. Follicular waves are present in these pubertal animals but fecundity starts later on, since ovulation does not yet occur at this age. Ovulation is only starts from four to five years of age. Social hierarchy might be one of the triggers to inhibit ovulation at this age. Suppression of subordinates might lead to social stress, which can have a negative impact on reproductive performances (Hermes et al. 2006; Metrione et al. 2007). As in the horse and most other mammals, ovulation occurs after a single pre-ovulatory LH peak. The size of the pre-ovulatory follicle varies between the different rhinoceros species. In captive white rhinoceros, Graafian follicles reach a preovulatory size of 30-34 mm (Radcliffe et al. 1997; Hermes et al. 2007; Hermes & Hildebrandt 2011). Follicular growth rates, as measured in two induced oestrous cycles, were calculated at approximately 2 mm per day (Hildebrandt et al. 2007). Graafian follicles of captive black rhinoceros reach a preovulatory size of 47-51 mm. Once the dominant follicle reaches 35 mm in diameter, the follicular growth rate is approximately 3 mm per day (Radcliffe et al. 2001). The formation of anovulatory, haemorrhagic follicles, which exceed Graafian follicle size by 10 to 30 mm, are known to occur in captivity in all four rhinoceros species and are of great concern for their negative effects on fertility (Hermes & Hildebrandt 2011). In oestrus, female rhinoceros start to urinate small volumes, show tail-lifting and accept male rhinoceros in their close vicinity. Some of the females show a standing reflex to be mounted (Radcliffe et al. 1997). Local changes are also present in the genital tract of the females during oestrus. Vulvar swelling is classified as an indicator for oestrus in the female rhinoceros. Swelling is not diffuse but more 'bubble-like' (Carter 2007). There is also a colour change of the vaginal mucosa during oestrus: it becomes hyperaemic and red during oestrus and is pale pink during anoestrus. Colour changes are seen from two days before until three days after oestrus (Radcliffe et al. 1997). This is a fairly reliable, non-invasive technique to assess oestrus.

Within the different rhinoceros species, different oestrous cycle lengths have been observed, i.e. in the white rhinoceros 28 days (Bertschinger 1994), 30-35 days (Hindle et al. 1992; Brown et al. 2001) up to 65-70 days (Schwarzenberger et al. 1998) and in the black rhinoceros 27 days (Schwarzenberger et al. 1993; Garnier et al. 2002). Only in the shorter 30-35 days cycle, the captive white rhinoceros appears to be fertile; the longer cycle of 65-70 days, which is also seen in captive rhinoceros, has been associated with reproductive aging in older females (Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001).

The gestation length varies between the rhinoceros species, with the average being 15-16 months for the white, Sumatran, Indian and the black rhinoceros and the Javan at 16-19 months (Hermes et al. 2007). Pregnancy in captive rhinoceros can be diagnosed by the measurement of elevated progesterone and progestogen levels in blood, urine and/or faeces (Radcliffe et al. 1997) at about 3-5 months after conception. In free-roaming rhinoceros, the non-invasive method of faecal sampling is commonly used. In pregnant animals, the progesterone metabolite levels (5 α -pregnan-3 β -ol-20-one) are significantly higher than in non-pregnant and postpartum animals (Goot et al. 2013). The collection of freshly produced faeces as the predictive tool of the oestrous cycle is the most appropriate method. Faecal extracts can be measured for immune-reactive progesterone metabolites using an enzyme immunoassay (EIA) for 5 α -pregnan-3 β -ol-20-one. These metabolites have been shown to provide reliable information regarding to reproductive steroid hormone pattern by reflecting total progestogens in different mammalian species (Szdzyu et al. 2006; Ahlers et al. 2012). Faeces samples need to be freshly collected, stored frozen or lyophilized afterwards, and can be analysed eventually or stored for a long time. Only very few labs offer these hormone analyses on faeces. In addition, early pregnancies of 1-3 months are more difficult to detect with faecal progestogen metabolite measurements, because they sometimes level luteal phase values and there is no clear cut-off value (Hildebrandt et al. 2007; Goot et al. 2013). In some cases, inconsistency in plasma and faecal hormone levels is present until 4-5 months of pregnancy, which makes it more difficult to differentiate early pregnancy from normal cyclicity (Hermes et al. 2009b).

Blood and urine sampling in wild animals is more invasive and can only be performed in restraint or immobilised animals in the wild or well-trained animals in captivity.

Ultrasound has also been used to detect pregnancy in captive rhinoceros. Pregnancy diagnosis can be done starting from 15 days post-ovulation by visualization of the spherical embryonic vesicle. The embryo itself can be visualized as early as day 23 post-ovulation followed by heartbeat detection at day 26 (which is in accordance with the timing of the development in domestic horses). Mobility of the embryo during early pregnancy is seen both in the rhinoceros as well as in the domestic horse (Radcliffe et al. 1997; Hermes & Hildebrandt 2011). Similar to the horse, the placenta is of a diffuse epithelio-chorial type, with large avillous areas, so called 'streets' (often along the larger blood vessels of the allantochorion). They are covered by a simple columnar epithelium (Benirschke & Lowenstine 1995).

1.2.2 Anatomy of the male genital tract

The stallion has two ovoid testes, which are located in a horizontal position in the scrotum between the hind legs. The total scrotal width varies between 10.3 cm and 12.7 cm, depending on age and season (Burns et al. 1984). Testicle size is on average 11 to 12 cm in length, 5 to 7 cm in width and an average weight of 225 g per testis (Amann 1981), with breed differences. The testes are positioned horizontally, with the caput epididymidis cranially and the cauda caudally, while the corpus epididymidis is located along the dorsomedial margin of the testis.

In the rhinoceros, both testes are positioned horizontally in the scrotum, between the hind legs, comparable to the orientation in the stallion. Based on 24 ultrasounds of 21 male white rhinoceros, the mean testicular and epididymal diameter is 6.5 ± 0.3 cm and 2.8 ± 0.1 cm, respectively (Hermes et al. 2005). Due to the thick scrotal skin and the dense tunica, it is difficult to examine the testicles by palpation. Similar to the stallion, the rhinoceros has a penis of the musculocavernous type. In rest, it is pointed in caudal direction, even when the animal is urinating. Hence, the urine stream is always directed caudally. The preputial orifice is located just caudally to the umbilicus. Only when erect, the penis points in a cranial direction. Similar to the tapir but different to the stallion, the rhinoceros has penile flaps on both lateral sides of the penis. The glans penis has a typical, mushroom-like shape, but the tip is more gracile and much smaller. This shape together with the penile flaps suggests that the male rhinoceros is a cervical ejaculator (Hermes & Hildebrandt 2011). Accessory sex glands present in the stallion are the bulbo-urethral glands, the prostate, the seminal vesicles and the ampullae ductus deferentes (Little & Woods 1986). Bulbo-urethral

glands, seminal vesicles and prostate are comparable to those of the stallion (Schaffer et al. 1990). In rhinoceros, there are no ampullae at the end of the deferent ducts. Histologically, the deferent duct consists of an attenuated simple cuboidal epithelium with occasional gland-like extensions off the lumen and with a thick muscular coat (Hermes et al. 2005; Hermes & Hildebrandt 2011).

1.2.3 Semen

In rhinoceros, there is a positive correlation between semen quality (viability, morphology and motility) and pregnancy rates (Hermes & Hildebrandt 2011). Semen quality is species-dependent, but is also affected by the semen collection method (Schaffer & Beehler 1988; Hermes & Hildebrandt 2011) (Table 1). Inbreeding and increased age of the male cannot be ruled out as possible negative influences on semen quality (Hermes et al. 2005). Apparently, semen quality in rhinoceros is also influenced by the social status of the bull in the group. If the bull switches from a subdominant to a dominant position in another herd, this can have a clearly positive effect on its fertility (Hermes et al. 2005). Libido, spermatogenesis and mating behaviour of bulls are positively correlated with testosterone levels. In the wild, territorial bulls have higher testosterone levels than non-territorial bulls (Rachlow et al. 1998; Kretzschmar et al. 2004).

Table 1. Semen parameters of the rhinoceros (electro-ejaculation), Przewalski horse (electro-ejaculation) and the horse (artificial vagina) (Stover et al. 1981; Juhász et al. 2000; Hermes et al. 2005; Roth et al. 2005; Miller & Fowler 2011; Luther 2016)

Parameter	White rhinoceros (<i>Ceratotherium simum</i>)	Black rhinoceros (<i>Diceros bicornis</i>)	Przewalski (<i>Equus ferus przewalskii</i>)	Horse (<i>Equus caballus</i>)
Collection method	Electro-ejaculation	Electro-ejaculation	Electro-ejaculation	Artificial vagina
pH	8.0	7.9 - 9.5	7.1 - 8.2	6.7 - 7.5
Concentration (x10 ⁶ /mL)	9 - 152	0 - 66400	200 - 4700	178 - 335
Abnormal cells (%)	31 - 38	26 - 96	77	33 - 53
Total motility (%)	27 - 88	0 - 90	30 - 70	53 - 76
Progressive motility (%)	Cat 1: > 75 (n=21) Cat 2: 50 - 75 (n=5) Cat 3: < 50 (n=8)	0.0 - 3.5	30 - 60	53 - 68

The progressive motility in the white rhinoceros was divided into different categories of quality by Hermes et al. (2005) (34 (n) ejaculates of 21 animals).

1.3 Reproductive pathologies in the rhinoceros

The breeding of captive rhinoceros provides a gene pool of valuable animals as a back-up source for wild populations. However, the natural breeding of rhinoceros in zoos continues to be problematic (Foose & Wiese 2006). One of the main problems of the captive breeding of rhinoceros is the high incidence of prolonged periods of anoestrus in females, with more than half of these females remaining acyclic. This occurs both in old and young females (Hermes et al. 2004; Hermes et al. 2006;

Hermes et al. 2007). In these young females, regular follicular waves without ovulation of the pre-ovulatory sized follicles is seen (Radcliffe et al. 1997; Roth et al. 2004; Stoops et al. 2004). Another cause of the prolonged infertile cycle might be early embryonic death. Embryonic death can be associated with prolonged maintenance of the corpus luteum, the so called persistent corpus luteum. This feature has also been described in the domestic cow (Kastelic et al. 1988; Diskin & Morris 2008) and horse (Bergfelt et al. 1992; Allen 2001a).

One of the approaches to improve fertility of captive rhinos is to increase exposure to other individuals of the same species. When female rhinoceros are translocated to other facilities, the effect of transportation as well as encountering new rhinoceros can be sufficient to initiate regular oestrous cycles. Likewise, the introduction of new males in a group can induce cyclicity in previously anoestrous females (Rachlow et al. 1998; Kretzschmar et al. 2004; Hermes et al. 2005). This indicates that socio-behavioural dynamics and/or pheromones influence the oestrous cycle and ovulation in the rhinoceros. Poor management and animal husbandry often contributes to irregular or inactive cycles (Carlstead et al. 1999; Hermes et al. 2004). However, it should be kept in mind that moving animals between different facilities cannot be used as a routine management procedure in captivity. Other reproductive pathologies are anovulatory and haemorrhagic follicles, which are common findings both in the white rhinoceros and in the domestic horse (Hermes et al. 2007; Cuervo-Arango & Newcombe 2012). Sometimes only subtle clinical signs of a possible reproductive pathology, such as vaginal discharge, are present in rhinoceros despite a prominent reproductive pathology of the uterus diagnosed later on necropsy. Recently, the increased application of ultrasound has facilitated earlier diagnosis and treatment of reproductive pathological conditions in the living animal. Pathologies, such as tumours and cysts of the endometrium and ovary, muco- and pyometra, and uterine leiomyoma, endometrial adenoma and adenocarcinoma have been reported to occur in rhinoceros (Hermes & Hildebrandt 2011).

Reproductive pathologies in male rhinoceros are less well-known. Penile oedema with penile prolapse, which prevents normal mating, has been described. In zoological institutions testicular fibrosis, testicular neoplasia and epididymal cysts have been incidentally discovered, in some cases with a negative influence on fertility (Portas et al. 2005; Portas et al. 2010; Hermes & Hildebrandt 2011).

1.4 Role of assisted reproductive techniques and their benefits in rhino breeding

Assisted reproductive technologies (ART) have been applied with great success in several domestic species. Nevertheless, researchers are repeatedly faced with the problem that the implementation of these techniques in wildlife is hampered by the distinct traits of each species. This precludes the easy transfer and application of this technology, despite it being refined in domestic animals over the past decades. To apply ovum pick-up (OPU), in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI), good knowledge of the female oestrous cycle and the anatomy of the reproductive tract of that specific species could be helpful (Hermes et al. 2009b). This remains a problem in wild species, such as the rhinoceros, in which characteristics of oestrous cycles are less well-defined and even differ between captive and free-ranging rhinoceros (Hermes et al. 2004; Hermes et al. 2006; Goot et al. 2013). To increase basic research efforts in these wild animals, more financial support is needed. Since wild animals generate less economic interest worldwide and because their wild state of being complicates easy collection of data, this presents as an ongoing problem.

Despite the potentially problematic species variation and implementation of ART in captive breeding management, ART still has several advantages. Assisted reproduction can avoid unnecessary risks during long transportation of living animals to increase animal numbers in a population. Moreover, there is a reduced risk of disease transmission by implementing ART. With the aid of reproductive techniques, there might also be an increased chance of breeding with sub- or infertile animals as well as with animals that are physically or socially unable to mate naturally. Although, depending on why they are subfertile, ART in subfertile animals should not be encouraged. Even post-mortem material from recently deceased animals can be used (e.g. epididymal semen or oocytes) as a source for ART (fresh, cooled or cryopreserved). However, since so little is known about these species, any interfering factor like sub- or infertility is bound to compromise any ART procedure to some degree.

The northern white rhino, with only two remaining individuals left, recently gained a lot of publicity with all kinds of crowd funding actions to develop on a short notice ART to help save this subspecies of white rhinoceros (conservancy 2017).

Researchers proposed a road map that, if successfully implemented, would ultimately lead to a new self-sustaining population of northern white rhinoceroses (Saragusty et al. 2016) (figure 1).

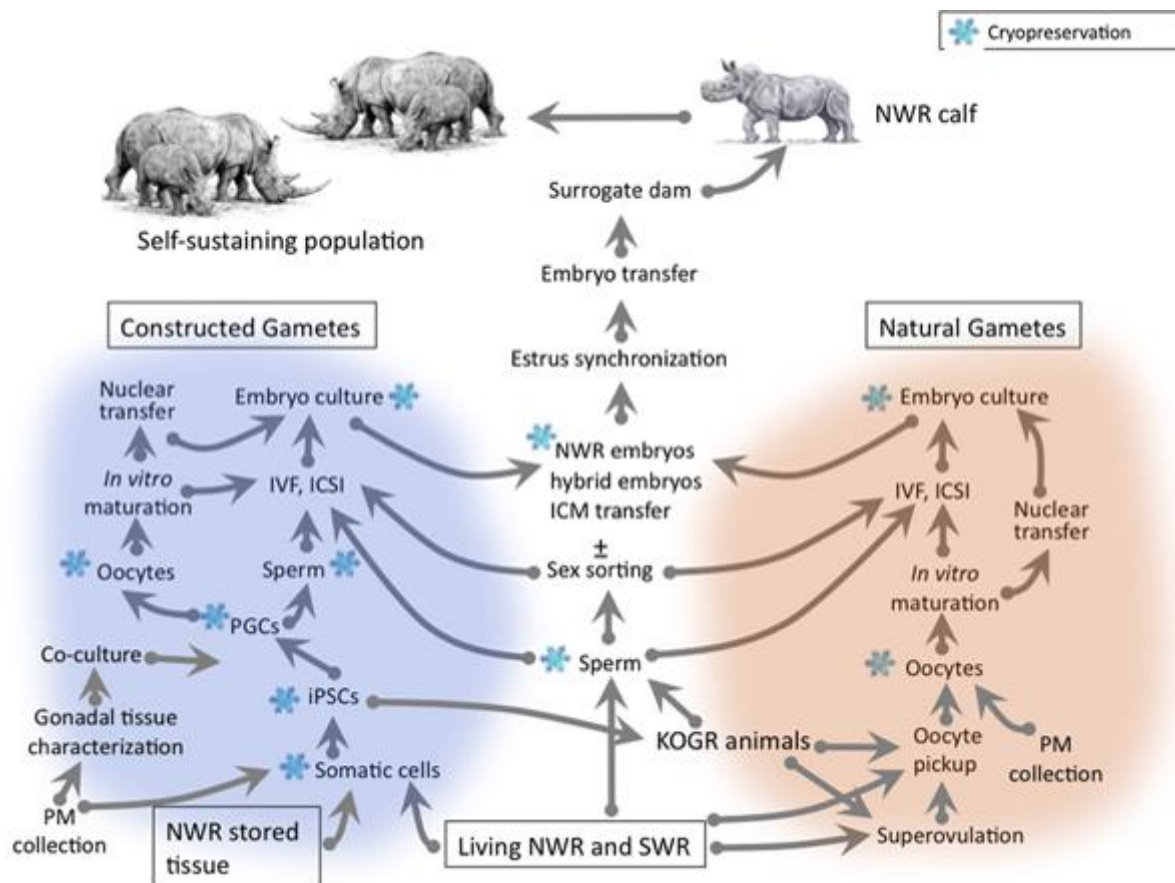


Figure 1. Road map of rewinding the process of mammalian extinction (Saragusty et al. 2016). Detailed are the resources and the flow of the process using natural gametes (right side of the diagram) or constructed gametes (left side of the diagram) leading to, what they hope, to live birth of a northern white rhinoceros (NWR) out of a surrogate southern white rhinoceros (SWR).

Most of the assisted breeding techniques used in wild animals have been extrapolated from techniques used in domestic animals. Techniques such as pregnancy diagnosis by ultrasonography, AI, embryo transfer (ET) and OPU can be applied in wild species too, provided that they are adapted to the anatomical configuration and reproductive characteristics of the species in question.

Animal restraint

Husbandry systems used in horses are very well accessible for routine use in equine ART. In wild animals such as the rhinoceros, this is more complicated. These

animals often need to be heavily sedated or immobilised to be able to work safely. In trained animals, chute conditioning with food may help the animals entering and remaining in a free-stall chute (Radcliffe et al. 1997). Other types of cage restraints may interfere and restrict the way of working (Schaffer et al. 1998b). In case of more invasive ART, such as ovum pick-up, the animals need to be fully restrained and anesthetized to facilitate handling (Hermes et al. 2009a).

Regulation of the oestrous cycle

Regulation of the oestrous cycle might be used for oestrus induction, ovulation induction or oestrus synchronization. A major impediment to the use of AI is the detection of oestrus for the optimum timing of breeding. Good observation of the animals assists in detecting natural oestrus, but oestrus synchronization might be a very important tool as well. In domestic species, protocols have been developed to synchronize and regulate follicle development, luteal regression and the time of ovulation (Bisinotto & Santos 2012). By controlling ovulation via synchronization, AI and ovum pick-up can be timed, although the latter one not necessarily needs to be timed.

In the domestic horse, ovulation synchronization programs are widely used. Oestrus synchronization is used as part of the insemination strategy (timed insemination) on larger stud farms or AI centres (Allen & Cooper 1975; Bergfelt et al. 2007; Handler et al. 2007; Squires & McCue 2007), and as such, a single ejaculate from the same stallion can be applied to inseminate several synchronized mares. To gain high success rates in equine ET, it is necessary that the recipient mare is synchronized according to the moment of ovulation of the donor mares (Raz et al. 2011). In this way, pregnancy rates of > 70 % can be achieved (Carnevale et al. 1987; McKinnon & Squires 1988; Jacob et al. 2012; Leemans et al. 2012; Vandenberghe et al. 2012). As for wildlife, general information about the reproductive cycle of wild animals is collected by non-invasive hormonal analysis (faeces, urine) and by observation of behavioural changes. But more invasive techniques, such as oestrus synchronization and ovulation induction, might provide aid for the successful implementation of AI programs in wildlife. Although few data were available on programs developed specifically for wildlife species (Pukazhenthil & Wildt 2004), the application of such

programs has been described in captive rhinoceros (Schwarzenberger et al. 1998; Hildebrandt et al. 2007; Hermes et al. 2012).

Oestrus synchronization and oestrus and ovulation induction have not yet been performed in the wild rhinoceros. Various combinations of follicle-stimulating hormone (FSH), equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), and gonadotropin-releasing hormone (GnRH) have failed to induce ovulation in the captive rhinoceros (Godfrey et al. 1990; Hermes et al. 2012; Hildebrandt & Hermes 2014). Other possibilities, although perhaps more complicated, are being investigated, i.e. hormone treatments based on the long term downregulation of ovarian activity using long acting GnRH implants combined with an injection of hCG at the end, or using synthetic progesterone (Chlormadinone Acetate; Synchrosin®, Werfft-Syntex, Vienna, Austria) followed by an injection of hCG (Chorulon®, Intervet, Boxmeer)(10000 IU). Doses and treatment intervals have been extrapolated from those used in domestic horses (Schwarzenberger et al. 1998). Although continuous cyclicity could not be induced, ovulation induction has been successful in white rhinoceros (Schwarzenberger et al. 1998; Hermes et al. 2012). In a study by Hildebrandt et al. (2007), a more simplified protocol induced ovulation during anoestrus with a GnRH analogue, deslorelin (Ovuplant®, Peptech, Melbourne, Australia). This short-term GnRH analogue was implanted subcutaneously on the day of insemination, in two oestrous cycles, resulting in ovulation and pregnancy (Hildebrandt et al. 2007). Due to the thick skin, it is difficult to remove the deslorelin implant. It is often left behind under the skin. Nowadays, the use of injectable hCG (Chorulon®, MSD, Boxmeer, the Netherlands) (10,000 IU) one day prior to AI is suggested instead of the deslorelin implant (Hermes et al. 2012; Hildebrandt & Hermes 2014).

Semen collection

Stallions can be trained to mount a phantom; this is obviously much more difficult in the rhinoceros. Male rhinoceros are either shy or aggressive, which can be dangerous as they become unpredictable, and hence might be difficult to handle or to train (Young 1967; Schaffer et al. 1990). Some researchers have tried to develop a modified artificial vagina to collect semen, based on the artificial vaginas used in stallions. Due to the size and anatomy of the rhinoceros penis and the lack of

appropriate sexual stimulation, the collection of a good-quality ejaculate has been challenging, resulting in ejaculates with a low concentration of spermatozoa and in most cases, a low progressive motility (Schaffer et al. 1990). Penile massage is a technique that is commonly used to induce ejaculation in primates, pigs and carnivores. In other large mammals, it is usually ineffective, although it can be used for semen collection in stallions (Crump & Crump 1989). In rhinoceros, it has been used to collect semen, although it is not the most effective way. This technique is often combined with rectal stimulation (massage or electro-ejaculation). The semen quality after penile massage is usually not as good as when semen is collected by other means, such as electro-ejaculation combined with rectal massage (Schaffer et al. 1990; Hermes et al. 2005).

Electro-ejaculation is commonly used method for semen collection, especially in wild species. In horses, this invasive and painful method is rarely used, since stallions are easy to train to mount a dummy for collection with an artificial vagina. In some cases however, electro-ejaculation is used as a terminal semen collection method, for example when stallions are no longer able to mount physically or stand up (Cary et al. 2004). Because it is difficult to work with wild animals without sedation or anaesthesia, electro-ejaculation with concurrent sedation is an acceptable method for semen collection in the rhinoceros species (Schaffer et al. 1990; Roth et al. 2005; Luther 2016). Probes are specially designed and adjusted to the different rectal anatomy of rhinoceros (Hermes et al. 2005; Roth et al. 2005). Typical rectal probes have longitudinal electrodes. Low electric stimuli are applied to the nerves of the accessory sex glands and vasa deferentia of the male rhinoceros, which are adjacent to the neck of the bladder and lie ventrally to the rectum (Schaffer et al. 1998a; Hermes et al. 2005). Electro-ejaculation in the rhinoceros is often combined and followed by manual massage of the pelvic and penile parts of the urethra (Schaffer et al. 1990; Schaffer et al. 1998a), which may result in urine contamination. Semen characteristics in southern white rhinoceros have been studied after electro-ejaculation (Luther 2016). The average rhinoceros ejaculate in Luther 2016, contained a total number of 1.1×10^9 spermatozoa (volume of $24 \pm 24\text{mL}$ x concentration of $83 \pm 96 \times 10^6 / \text{mL}$) that recorded a total motility at $82 \pm 8\%$ of which $28 \pm 23\%$ were progressively motile. Computer assisted sperm analysis (CASA) recorded velocities for VCL ($85 \pm 29\mu\text{m/s}$), VSL ($44 \pm 25\mu\text{m/s}$) and VAP ($69 \pm 30\mu\text{m/s}$, and kinematics at STR ($63 \pm 14\%$), LIN ($51 \pm 16\%$), ALH ($2 \pm 0.16\mu\text{m}$) and

BCF ($16 \pm 6\text{Hz}$). Structural analysis revealed that $73 \pm 10\%$ of the spermatozoa were viable (intact plasma membrane) and $76 \pm 4\%$ maintained acrosome integrity (eosin-nigrosin stain and spermac®). Ejaculates contained $62 \pm 14\%$ morphologically normal spermatozoa, CASA (CASMA) measured sperm head lengths at $5.5 \pm 0.17\mu\text{m}$ and width $2.9 \pm 0.19\mu\text{m}$ (total head area of $14.8 \pm 1.43\mu\text{m}^2$) of which $36.3 \pm 0.59\%$ is covered by an acrosomal cap. As described in horses, there might be a chance of retrograde ejaculation during electro-ejaculation when attempted under general anaesthesia (Cary et al. 2004). This may affect semen quality.

As an alternative for electro-ejaculation or to apply electro-ejaculation in the gentlest possible way, the administration of hormones, which induce contractions of smooth muscle, such as oxytocin or prostaglandins, can be used. Oxytocin treatment to enhance smooth muscle activity in the ductus deferens, prior to electro-ejaculation has resulted in increased numbers of spermatozoa in the ejaculate of bulls (McDonnell et al. 1987a; Berndtson & Igboeli 1988). Prostaglandin F₂ α used prior to semen collection may also influence smooth muscular contraction and consequent semen collection. In stallions however, the administration of prostaglandin F₂ α has been linked with inconsistent results (McDonnell et al. 1987a). In rhinoceros, this treatment has not been described yet.

Imipramine, a tricyclic antidepressant of the dibenzazepine group, with both central and peripheral effects on neurotransmission, has been used in stallions with ejaculatory dysfunction. Imipramide reduces the ejaculatory threshold. Ejaculation can be induced by alpha 2 agonists, such as medetomidine and xylazine, who stimulate peristalsis of the vas deferens and thereby facilitate emission of an ejaculate with a higher sperm concentration. Positive effects on erection and ejaculation were achieved in the stallion, but results were variable (McDonnell et al. 1987a; McDonnell 2001). It is important that the stallion was not stressed and kept undisturbed. In some cases, the imipramine treatment has been combined with injections of xylazine; in other cases ejaculation has been induced with xylazine alone (McDonnell & Love 1991). The induction can be with or without sexual prestimulation. The induced ejaculates are of lower total volume, higher concentration, lower gel volume, higher total numbers of spermatozoa and lower pH than normal ejaculates after copulation (McDonnell et al. 1987b; McDonnell & Odian 1994). It remains to be determined if this method is equally suitable for the male rhinoceros.

Post-coital semen collection could be a good alternative in cases where animals are difficult to train for mounting a phantom. Although it is not the best (semen is often mixed with other vaginal fluids/cells) or most practical method, post-coital semen collection can be used in some cases, more specifically when the female is kept in a closed environment and can be darted. The semen samples collected via this method represent a sample of a natural ejaculate, whereas the small volumes of fluid emitted during methods like manual stimulation or electro-ejaculation may not consist of the appropriate mixture of seminal fluids (O'Brien & Roth 2000).

Collection of epididymal spermatozoa is a terminal procedure in animals with irreparable conditions, such as complicated fractures, soon after (natural or induced) death occurred or directly after castration. It also might be of great value to maintain genetic diversity in a gene bank for endangered species.

The main two different techniques of epididymal sperm collection, retrograde flushing and floating method, are commonly used in domestic horses (Cary et al. 2004; Roels et al. 2014). These techniques have also been used in other species, such as goats, dogs, cows and humans (Marks et al. 1994; Sharma et al. 1997; Martins et al. 2007). In rhinoceros, it has also been used as a semen collection technique, especially in cases where the animal had died (Williams et al. 1995; O'Brien & Roth 2000). Testes were removed 1-3 hours after death and semen was collected approximately 3-30 hours post-mortem (O'Brien & Roth 2000). In this specific study, sperm motility of 60% (in black rhinoceros) could be achieved by warming samples to 37°C. More than 80% contained cytoplasmic droplets and 60% had an abaxially placed midpiece. This last characteristic is also seen in stallions (O'Brien & Roth 2000). Williams et al. (1995) recovered 80-85% motile sperm in white rhinoceros. However, the number of reported cases is too small to set a standard for this method, but with the rising increase of man-induced deaths, such as poaching, this method may become more important in the future.

Semen processing

The use of frozen semen in domestic animals like horses and cattle has been successful for years. Cryopreservation with an suitable extender for semen has proven successful in cattle and horses since the fifties of last century (Barker & Gandier 1957; Foote 2002; Allen 2005). The ability to freeze semen made it possible

to spread valuable male genes from excellent sires during and also after their sports career. In addition, AI with either fresh, cooled or frozen semen results in a reduced transmission of venereal and other diseases (Foote 2002).

In rhinoceros however, the application of frozen semen has only been successful since the beginning of the 21st century. In the rhinoceros, cryopreservation of semen is quite similar to that in the stallion. It has been found that rhinoceros sperm may survive the cryopreservation process as evaluated by motility and membrane integrity. In a study (Hermes et al. 2005) , the samples were immediately after collection of semen diluted (1:1) with pre-warmed (37°C) cryoextender BC (Berliner Cryomedium). Semen extender BC is based on a buffer solution containing TES, TRIS, fructose and lactose, and supplemented with egg yolk (~16%), DMSO (~6%) and α -tocopherol (20 IU/ml). BC extender was chosen in these experiments, because of its proven efficiency in preserving semen from a large variety of endangered species. In this study of Hermes et al. 2005, 12 white rhinoceros were included and in total 14 ejaculates (motility $\geq 50\%$) were frozen. Samples diluted 1:1 were centrifuged (800 x g) for 10 minutes at room temperature (20-23°C) to eliminate seminal plasma from the ejaculate. After removal of the supernatant, the samples were re-extended with BC to four times the native sample volume. The samples were equilibrated for 2 hours at 4°C, frozen in 0.5 mL straws, 2 cm over liquid nitrogen vapour for 15 minutes before being plunged into liquid nitrogen. For evaluation and usage, straws were thawed in a 38°C water bath for 60 seconds and evaluated after 10-15 minutes of incubation at 37°C. In a comparative trial, ejaculates of five males were treated as described above but diluted with four different extenders, i.e. Berliner Cryomedium, Biladyl (supplemented with egg yolk and DMSO), Gent (egg yolk-based) and Kenney (supplemented with egg yolk and DMSO). Berliner Cryomedium (BC) maintained sperm quality better than the other extenders (Hermes et al. 2005).

The first successful AI in a rhinoceros was reported in 2007, using fresh semen (Hildebrandt et al. 2007; Hermes et al. 2009b). Only in 2009, AI with frozen semen resulted in the birth of the first living rhinoceros calf (Hermes et al. 2009b). In this particular case, the directional freezing technique was used (Hermes et al. 2009b; Reid et al. 2009). Two inseminated cycles were necessary to obtain a pregnancy. In the first (unsuccessful) insemination, a dose of approximately 135×10^6 motile sperm cells was used, whereas in the second (successful) attempt the dose was increased to approximately 500×10^6 motile sperm cells. In the latter case, the semen was

collected using electro-ejaculation, using a customized probe, 125mm long with a diameter of 105 mm and three longitudinal, slightly raised electrodes. The semen was immediately extended with isothermal BC at a ratio of 1:1. The extended semen was chilled slowly over about 2 hours inside an isothermal water bath stored at 4°C. The chilled semen was packaged into 8 mL and 2.5 mL Hollow Tubes and frozen using the MTG-516 apparatus (IMT Ltd., Nes Ziona, Israel). The frozen samples were kept under liquid nitrogen till the moment of insemination. For thawing, the samples were first exposed to air at room temperature (22-23°C) for 60 seconds, and then plunged into a water bath at 37°C for 30 seconds.

In a study by Reid et al. (2009), liquid nitrogen vapour (LN vapour) freezing was compared to multithermal gradient directional freezing in ejaculates of sixteen white rhinoceros. All of them were electro-ejaculated and semen was diluted with cryoextender (TRIS, lactose, egg yolk, DMSO). Directional freezing resulted in a higher semen viability (5.6%) and progressive motility (34.7%) compared to LN vapour freezing.

Artificial insemination

The use of fresh and cooled semen for AI is spread throughout most domestic animal species, such as horses, donkeys, swine, cattle, dogs, sheep and goats, and the cat being an exception. Up till now, in breeding programs, better results have been obtained by natural breeding than by AI. Besides studbook regulations and restrictions, sometimes, it is geographically impossible to bring the male and female animal together.

In zoos and game farms, breeding often happens naturally, which yields the best results but is never without the risk of injuries. These risks, however, are no greater than the risks associated with immobilisation for ART. Breeding healthy, fertile animals evidently leads to the best breeding results and maintains the best genetics in a population. However, sometimes, assisted breeding techniques might be useful in animals with acquired subfertility (Blyde 1997), as this is often the case in rhinoceros. Because of the low number and genetic variety of rhinoceros, breeding animals with acquired subfertility could be of additional value to preserve genetics. Hereditary subfertility, if known at all, should however be avoided in an already compromised genetic population like the white rhinoceros.

Artificial insemination in the rhinoceros represents an anatomical challenge due to the firm tortuous cervix of the female. To overcome this problem, a rhinoceros-specific AI-catheter has been developed (Hermes & Hildebrandt 2011). Artificial insemination with fresh semen has been executed in captive rhinoceros within zoological institutions (Hildebrandt et al. 2007). This is possible when both fertile male and female are on the same location, or within an acceptable geographical distance allowing preservation of semen during transport. Electro-ejaculation is usually followed by AI, when the female is anesthetized, examined and prepared for the insemination.

In case of wide geographic distances between the animals, or when the sire has died or has become unavailable for mating, AI can be done with frozen semen too. Semen collected after electro-ejaculation is diluted before cryopreservation. The use of frozen semen has led to one successful pregnancy after two inseminations in two different cycles (Hermes et al. 2009b).

Modern ART

Other techniques, like OPU, ICSI and cloning, have been used in rhinoceros as well, but so far with limited success (Hermes et al. 2007; Hermes et al. 2009a). Recent studies even focus on fibroblast cell lines, stem cell markers and production of stem cell lines (induced pluripotent stem cells) for future benefits in *in vitro* production of viable gametes and eventually live birth calves (Amato et al. 2009; Korody et al. 2017; Gomez et al. 2018). Although resources are limited in endangered animals, transfer of modern ART from domestic animals can lead to positive results.

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CHAPTER 2

SCIENTIFIC AIMS

The general aim of this research is to test the hypothesis that game-ranched African white rhinoceroses, even when regularly dehorned, can be used as a model for conservation of wild and captive populations. Breeding success in captivity, as described in the introduction (**Chapter 1**), is low and numbers of wild white rhinoceros are declining with a poaching rate of 1000 each year for the last five years close to or already exceeding the birth rate. Population numbers worldwide are estimated to be less than 20,000 according to the biodiversity management plan for the white rhinoceros in South Africa 2015 – 2020. To test our hypothesis, we studied the world's largest white rhinoceros game-ranch, located in South Africa, with around 1500 individuals present. Due to safety precautions, the anterior and posterior horn of the rhinoceros in the study are repeatedly trimmed in order to prevent poaching. Horns are safely stockpiled in banks for future purposes.

In order to realize the general aim, specific objectives were formulated as follows:

1. To determine breeding success of game-ranched rhinoceros we analysed the reproductive performances during an eight year study of 1.354 animals (**Chapter 3**).
2. To question whether routine dehorning has an influence on reproductive performances. Regular horn trimming is nowadays part of the anti-poaching management, with horn trade as a possible incentive for private owners. We studied the growth rate of the posterior and anterior horn and investigated whether these management interventions influenced the overall well-being based on reproductive health (**Chapter 4**).
3. To investigate the association between faecal androgen metabolite concentrations and reproductive performance in rhinoceros bulls. We studied age differences in testosterone levels and looked into social structures of males that are part of nowadays breeding success in captivity (**Chapter 5**).
4. To perform opportunistic breeding examinations during dehorning and translocation procedures to detect and evaluate reproductive tract pathologies. Additionally we compared detection of (early) pregnancy by ultrasound with faecal progestogen measurements (**Chapter 6**).

CHAPTER 3

Reproductive performance parameters in a large population of game-ranched white rhinoceroses (*Ceratotherium simum simum*)

Modified from:

Ververs C., van Zijll Langhout M., Hostens M., Otto M., Govaere J., Durrant B., Van Soom A. (2017). Reproductive performance parameters in a large population of game-ranched white rhinoceroses (*Ceratotherium simum simum*). PloS ONE 12(12): e0187751.

Abstract

The population of free-roaming white rhinoceroses (*Ceratotherium simum*) is under serious threat. Captive breeding of this species is therefore becoming more important, but this is challenging and often not successful. Obtaining reproductive reference values is a crucial aspect of improving these breeding results. In this study performed between 2008 and 2016, reproductive performance was analysed in 1,354 animals kept in a 8000 hectares game-ranch environment. Descriptive statistics of this captive population showed an average annual herd growth (%) of 7.0 ± 0.1 (min -9 – max 15). Average calving rates were calculated as an annual calving rate of 20% and biennial calving rate of 37% adult females calving per year. Females had a median age of 83.2 months at first calving (IQR 72.9 – 110.7) and inter-calving intervals of 29.2 (IQR 24.6 – 34.8) months. Furthermore, translocations of animals did not interfere with reproductive success in terms of inter-calving periods or age at first calving. Multivariate models showed a clear seasonal calving pattern with a significant increase of the number of calvings during December – April when compared to April – December. Our results did not show any significant skewed progeny sex ratios. Weather observations showed no significant influence of rain or season on sex ratios of the calves.

Introduction

The white rhinoceros (*Ceratotherium simum*) is considered to exist of two subspecies: the southern white rhinoceros (*Ceratotherium simum simum*), and the much rarer northern white rhinoceros (*Ceratotherium simum cottoni*). The southern white rhinoceros was exemplary for a major conservation success story. With numbers as low as 50 in the wild in the early 1900s, this subspecies has recently increased to a population of almost 20,000 individuals (Amin et al. 2006; Emslie et al. 2012). South Africa represents the major habitat for the southern white rhinoceros, conserving 16,255 individuals in the wild in 2007 (Ripple et al. 2015). However, poaching has increased dramatically since 2008, due to a huge demand for rhino horn, and is causing a rapid decline in wild rhinoceros populations and more specifically, of southern white rhinoceros (SWR) numbers in South Africa. Dwindling population numbers in the wild are mainly due to the inherent value of horn. If the current population numbers continue to diminish at the present rate, with over 1,200 southern white rhinoceros being poached in South Africa alone in 2015 and over 1,000 in 2016, captive breeding programs may be crucial to prevent extinction. Endeavours to breed white rhinoceros in captivity have led to growth and expansion of population numbers in the past with improved genetic diversity and an increased economic return of investment (Garnier et al. 2001).

Most reports on reproductive performances in zoological institutions have been about individuals or small groups of rhinoceros. Wild-caught southern white rhinoceroses will readily breed in captivity if given appropriate space and food, and if other female rhinos of breeding age are present. However, for reasons that are currently not understood, the rate of reproduction is extremely low among captive-born southern white females (Swaigood et al. 2006). One of the reasons might be, that in captivity, females are frequently bred for the first time at an older age and that the social hierarchy in smaller groups is often compromised (Hermes et al. 2006; Metrione et al. 2007). Captive populations kept outside their natural habitat also show a high incidence of prolonged periods of anestrus, with more than half of these females remaining acyclic (Hermes et al. 2006; Hermes et al. 2007) or without ovulation of preovulatory follicles (Radcliffe et al. 1997; Roth et al. 2004; Stoops et al. 2004). Furthermore, pathologies of the female genital tract, such as endometrial and ovarian cysts, muco- and pyometra, and uterine leiomyoma, adenoma and adenocarcinoma

have been documented to occur more in captive rhinoceros than in their free-ranging counterparts (Hermes & Hildebrandt 2011). Reproductive pathologies in male rhinoceros have rarely been noticed. Penile edema with prolapse, which prevents normal mating, has been described (Hermes & Hildebrandt 2011). Testicular fibrosis (age related), testicular neoplasia and epididymal cysts have been incidentally diagnosed in male rhinoceros at zoological institutions (Portas et al. 2005; Portas et al. 2010; Hermes & Hildebrandt 2011). However, reproductive pathologies may be diagnosed more often in captive herds due to more intense management (Ververs et al. 2015). Adverse physiological effects due to external and internal stimuli, such as diet, metabolism, disease, stress and environment may contribute to a low reproductive success rate in captivity. In fact, only 50% of all captive females reproduce successfully, and only 38% of the females born in captivity have produced offspring (Swaigood et al. 2006; Metrione & Harder 2011). In these captive-born a diet-related loss of fertility has been reported as well (Tubbs et al. 2012; Tubbs et al. 2016). Interestingly, male-biased sex ratios at birth (53 - 59%) have long been observed in the captive rhino populations (Dennis et al. 2007).

It is generally accepted that small and isolated populations, like the populations kept in zoos, are vulnerable to stochastic factors (Garnier et al. 2001), and may suffer from biased sex ratios, fluctuation in individual reproductive success (Foose & Wiese 2006), and increased inbreeding. The ability to maintain genetic diversity has been studied in detail in wild species, and the effective population size is considered to be 50 to preserve them from short-term genetic risks, but 500 animals are required to maintain long-term adaptability (Soulé 1980). The same may hold true for reproductive efficiency : many *in situ* and *ex situ* programs for rhinoceros taxa are reporting poor growth rates of the population due to declining reproductive success (Mills et al. 2006). Here we postulate that managing small populations of rhinoceros will decrease reproductive efficiency, and that a single meta-population together with large home range size may be necessary for optimal reproduction.

This study analysed reproductive features in a game-ranched population of southern white rhinoceros with 1,354 individuals that exhibited natural behaviour, providing valuable insight into the reproductive performance of confined, game-ranched rhinos. The data generated from this study will hopefully contribute to better understanding

and increase breeding success in captive and non-captive white rhinoceroses populations.

Materials and methods

Breeding premises and animals

The study animals ($n = 1,354$) were part of a confined private game-ranched southern white rhinoceros breeding herd and were kept in large breeding camps (9ha per adult animal). The study was conducted with permission of the owner. The game-ranch was located in a semi-arid climate, with high summer temperatures and short, cool, dry winters with frost. The mean annual precipitation was 530 mm, with most rainfall occurring during the summer months. Average midday temperatures for the area ranged from 18°C in June to 29.5°C in January. The region is the coldest during July when the temperature drops to 0°C on average during the night. Access to drinking water was ensured throughout the year. The facility consists of several adjoining properties divided in different breeding camps with 25-70 animals per camp depending on its size and location. To prevent overpopulation, sub-adults (2.5-6 yr) were removed from natal camps and placed in new camps to establish new breeding herds. This allowed population control and prevented inbreeding by creating new gene pools in each breeding population. In addition, new arrival adult bulls were introduced to the herds from outside the facility. Each breeding camp consisted of 1-3 adult breeding bulls with 25-30 adult females, 10-25 calves and 10-25 sub-adults. Each breeding camp was divided into two sections to allow rotational grazing over the summer months, and rhinos were given additional feed when natural grass was limited. Supplementary feed consisted of alfalfa (8 kg) and pellets (5 kg) delivered to each rhino daily during the dry winter season (June-August), then in decreasing amounts until adequate rainfall allowed the return to a fully natural grazing diet. Additional feeding during the winter was designed to maintain the reproductive condition of the animals and assure their general well-being. General additional husbandry requirements, including adequate shelter, shade, mud baths and rubbing post were also available within each camp. Natural mating occurred without human intervention other than controlling the breeding camp numbers and monitoring the carrying capacity of the field and camp with the minimal surface per rhinoceros set at 9 ha per animal. The rhinoceros were allowed to create their own natural hierarchy

and herd structures, as in free-roaming conditions. An ID-microchip (Identipet) was applied in the left neck of each rhinoceros, and notches were applied in both ears for identification from a distance. A permanent full-time veterinarian was present on the premises for veterinary intervention when needed.

When observed, mounting behaviour was recorded. Oestrous females showed obvious signs of mounting such as scuff marks on their flanks and back from the legs of the bull. The identity of the bull and the cow were recorded to monitor the fertility of each bull and to predict the possible parturition dates for the cow. Each calving was recorded either early in the morning or late in the afternoon.

Animals originated, both from free roaming national, provincial and private game reserves. Animals had been trans-located from different areas, private and national parks into the game-ranch facility. Animals were always darted and fully anesthetized before partially reversed and loaded into crates for transport. During long transport animals were kept tranquilized with acuphase and azaperone until upon arrival. Females with a calf at foot were trans-located in separate crates and reunited upon arrival. Animals were always released straight into a new herd, without any quarantine period. To reduce stress and avoid running through fences, feces found in translocation crates was put in every corner of the new camp. We analysed subsequent calving rates during the first 16 months after translocation to the facility, to determine if translocation affected possible pregnancies. After these 16 months, fertility was no longer linked to translocation.

The game-ranch was conducted in accordance with guidelines presented by the South African Development Community (SADC). The Rhino Program of the SADC aims to maximize the population, ensuring the welfare of the animals as well as long term genetic and demographic diversity. The annual population growth rate reflects the reproductive health of each population and the ability of the individuals in the population to reproduce under each management method. This can be defined as a number of key indicators that were used to determine population performance and reproductive health. The analysis reveals factors involved in population performance either above or below the internationally accepted minimum annual growth rate of 5% for rhinoceroses (Du Toit 1998). The calculation of the growth rate excludes translocations in and out of the population during the assessment period.

Statistics

Data recording started in 2008 when a first group of southern white rhinos arrived at the breeding facility, and continued for 8 years. Data were recorded on Excel spreadsheets (Microsoft, Seattle, WA). All entered data were verified with the original records. Animal data were checked for entry errors and edited if necessary according to the original records. The original data set included 562 lactations of 823 females calving from January 2008 through December 2015. The historical weather data were provided by the South African Weather Service (Service 2008-2016) and contained cumulative rain fall (mm) per month from the same time period recorded in the weather station Klerksdorp (26°89'80''S; 26°62'00''E at 1329m). Rain data were transformed into 4 categories using quartiles (0.0 – <0.8; 0.8 – <23.6; 23.6 – <52.2; 52.2 – 182.6 mm rain/month). Estimated month of conception was compared to rainfall data to assess the effect of precipitation on time (or month or season) of conception.

First, descriptive statistics were calculated to describe the southern white rhinoceros population. These variables included the herd growth, defined as the total number of reproducing animals minus the animals that died, divided by the total number of eligible animals. Eligible animals being defined as adult (≥ 5 years) animals present plus the number of adult animals that arrived that year. This way annual herd growth is based only on new-born animals within the facility and new arrivals were not counted as herd growth. The effect of the new arrivals on the growth of the population was expressed as the artificial growth parameter, which was calculated as the new arrivals divided by the eligible total of animals at the facility each year. The calving rate (CR) was defined as the percentage of females calving annually and biennially. Given the gestation period of the southern white rhinoceros of ca. 16 months, we found it more interesting to look at biennial calving rates. However, since most previous studies in wild populations refer to annual calving rates we calculated both to make a fair comparison with previous studies. The age at first calving was defined as the number of days between confirmed birth date and first calving date. The inter-calving interval (ICI) was defined as the number of days between two subsequent calvings. In addition to the standard barplots for herd growth and calving rate, scatter- and survival plots were constructed to illustrate, respectively, the variance in age to first calving and effect of translocation on calving. Interquartile

ranges (IQR) were calculated as a measure for variability, being equal to the difference between the upper and lower quartiles.

Finally, two multivariate analyses were performed in R (R Development Core Team, 2008) using the *lme4* and *fixed* package to model the number of animals born (family=Poisson) and sex ratio (family=binomial). Generalised mixed effect models were constructed using rainfall quartiles and month of birth nested within the year of birth as fixed effects. Final models were constructed by comparing the Schwarz's Bayesian information criterion and Akaike's information criterion (best fit closest to 0). Significance and tendency were declared at $P < 0.05$ and $0.05 < P < 0.1$ respectively. Data are reported as back transformed reduced model least square means with standard error unless indicated otherwise.

Results

Herd growth, calving rate, artificial growth, age at first calving, inter-calving interval and effect of translocation on subsequent calving

The first animals arrived at the facility in 2008. For the 8 years of the study an average of 70 ± 36 females was bought annually to expand the population and maintain genetic diversity. The average annual population growth rate (%) was 7.0 ± 0.1 (range -9 – 15). The average artificial growth rate (%), that includes the number of new arrivals, was 37 ± 32 (range 3-100). Herd numbers increased over the years with new arrivals and calves born (Table 1). Number of calvings between years was also significantly different.

Table 1: Population growth parameters from 2008-2015

Year	2008	2009	2010	2011	2012	2013	2014	2015	Total
Female arrivals	43	70	128	81	101	80	18	36	557
Male arrivals	18	27	19	50	65	49	3	3	234
Number of calvings	2	12	46	82	83	95	117	125	562
Number of deaths	2	27	12	19	14	25	72*	38	209
Cumulative population	61	143	324	519	754	955	1021	1147	1147
Birth/death ratio	1	0.44	3.83	4.37	5.93	3.88	1.63	3.29	-
Annual calving rate (%)	4.7	10.6	19	25	20	19	23	23	-
Biennial calving rate (%)	-	15.3	30	45	45	39	42	46	-
Herd growth (%)	0	-9	11	15	11	10	6	11	-
Artificial growth (%)	100	61	48	30	28	18	3	5	-

* In 2014 there was an exceptionally high number of animals that died (72), probably due to exceptional weather conditions (heavy rains and mild floods) and a suspected Clostridium outbreak.

Over 8 years ('08 – '15) a total of 562 calvings was recorded within the facility. When the previous calving dates of newly introduced females were available, these data were only incorporated into the sex ratio and seasonality analysis. During the study, 47.26% of the calves born were females and 52.74% were males.

The average percentage of adult females calving per year (Figure 1) is visualised as the calving rate. This represents the total number of reproducing females divided by the total number of eligible females (the total of eligible females present plus new arrivals and minus females that died). Over the course of the 8 year study an average of 71 ± 46 females each year produced offspring with a minimum of 2 reproducing animals in the first year of the breeding history and gradually increasing to a maximum of 125 reproducing animals out of 588 possible breeding females in 2015. The calving rate was calculated as an annual and biennial rate. The average annual calving rate was 20% and the average biennial calving rate 37%. Since the studied facility expanded each year we also analysed the artificial growth which was calculated by the number of newly bought animals divided by the eligible total of animals present. Artificial growth did gradually decrease and herd growth gradually increased during the study. Figure 1 shows the evolution of the different parameters that contribute to the population growth at the facility. During the study period only 7 stillbirths were recorded, however, due to extensive farm settings and wild predators the authors decided to include these animals into the dataset without further conclusions regarding total number of stillbirths and abortions. This, then, is a minimum estimate of stillbirths.

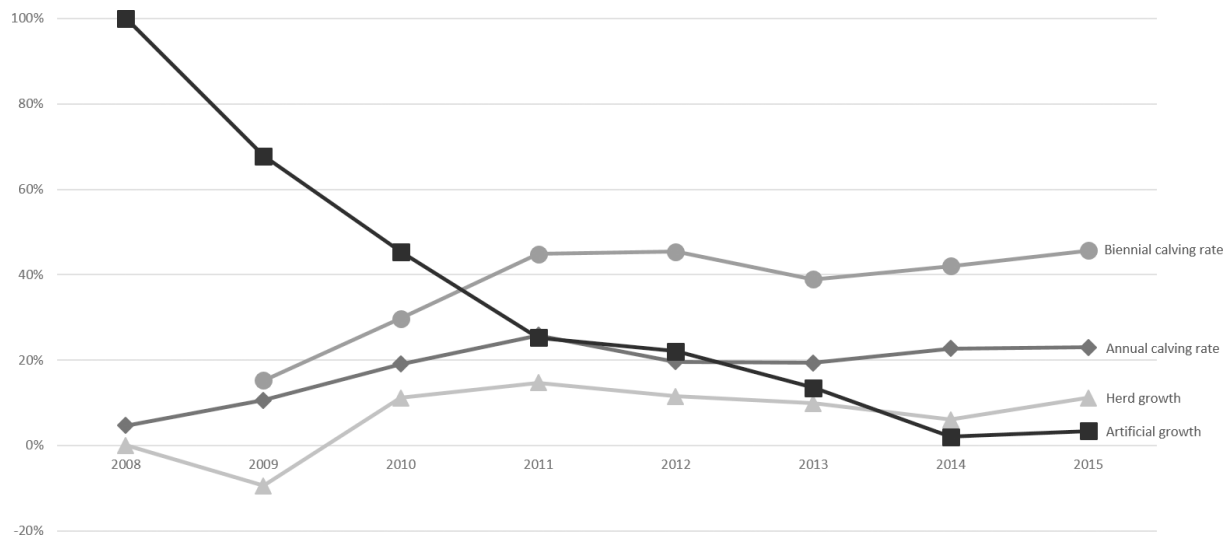


Figure 1. Comparison between the total herd growth, annual and biennial calving rate and the artificial growth. The y-axis shows the total percentage and the x-axis shows the time in years. In 2008 the breeding operation started with new arrivals which explains the 100% of artificial growth.

Figure 2 shows that the median age at first calving was 83.2 months (IQR 72.9 – 110.7). Records could only be taken from 2009 – 2016 since only these animals had a documented date of birth and hence a correct age at first calving. Other animals that calved only had estimated birth dates as they were bought into the facility. With a gestation period of around 16 months, animals from 2009 only calved at the end of 2010, as shown in figure 2.

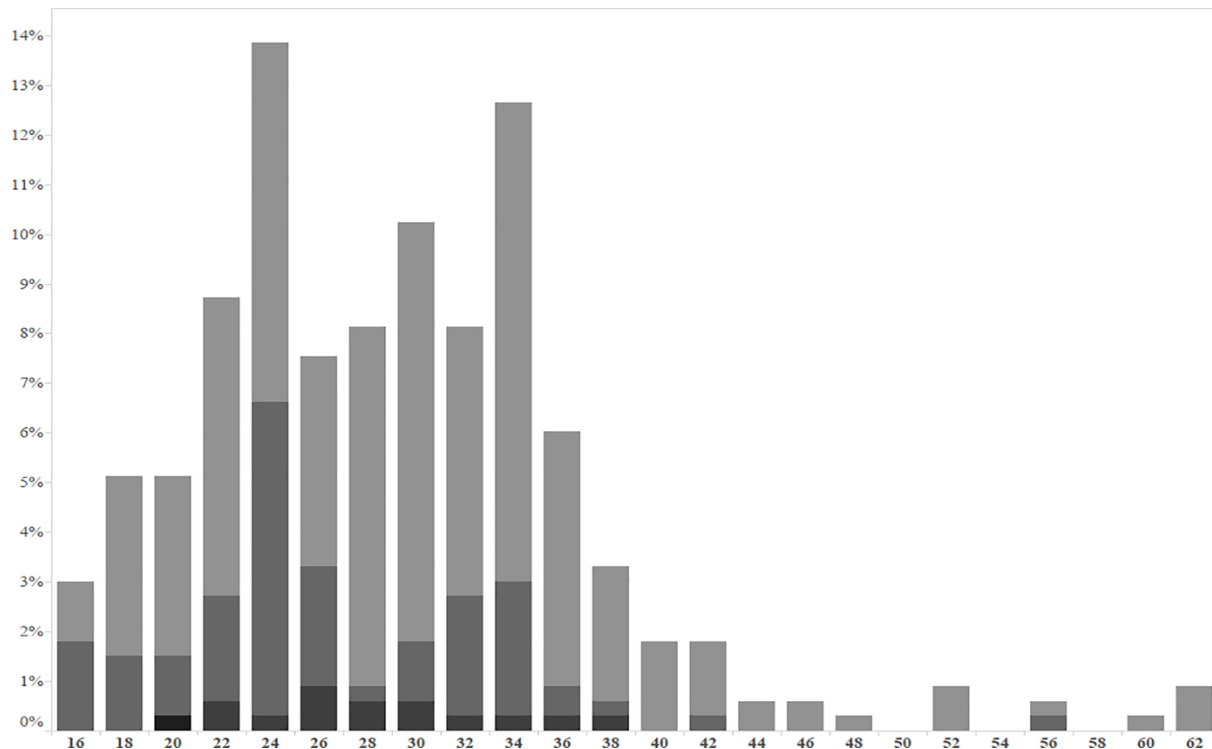


Figure 3. Histogram of calving intervals (in months). The x-axis shows the time interval (months) and the y-axis the percentage of animals. The difference in parity of the animals is indicated through grey scales with lighter bars (parity 1), medium bars (parity 2) and darker bars (parity ≥ 3) with lower parity animals having lighter bars.

Figure 4 shows the number of months after arrival in relation to the day of first calving at the breeding center. A large percentage of females calved within 16 months of arrival, indicating that these animals were pregnant during translocation. The slope of the graph indicates that the calving rate continues in the same pace before and after translocation. So either the method of translocation was not overly stressful or stress did not affect established pregnancies, as observed abortions were rare in recently translocated females.

By use of a Kaplan-Meier survival analysis we point out that 50% of the arrived females calved within 24 months of the arrival date. With a gestation period of around 16 months, this shows excellent fertility within the total herd. A slight drop in calving rates is seen in the first couple of months before and after arrival (Figure 4).

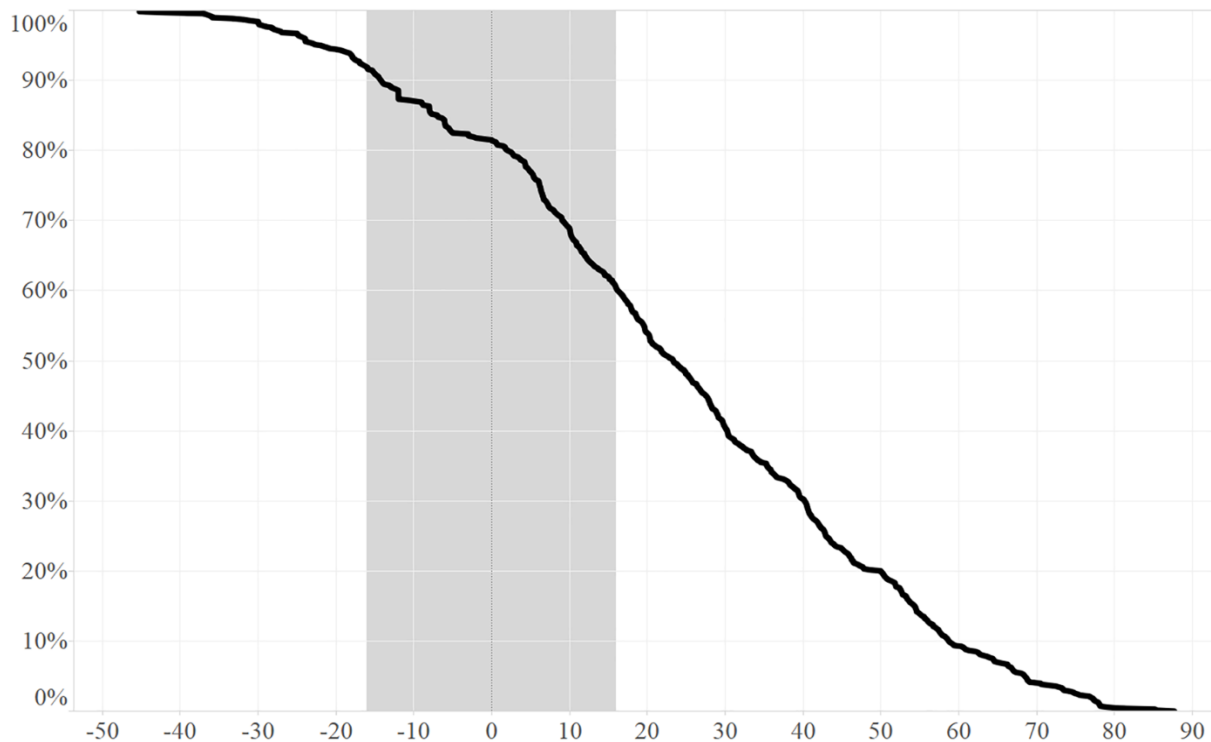


Figure 4. The percentage of animals calving relative to the month of arrival. The x- axis shows the time in months before and after the moment of arrival at the facility. The y-axis indicates the remaining population percentage to calve throughout the study period. The vertical grey bandwidth indicates the ± 16 months pre and post arrival interval.

Seasonal influences per month on number of births

The data showed a clear seasonal calving pattern (Figure 5) with a significant increase in number of calvings during December - April in comparison to the rest of the year ($P < 0.001$). Figure 5 shows a clear peak in the 562 calvings that took place at the study facility between 2008 – 2016. With the highest number of births per month being 28 in March 2015.

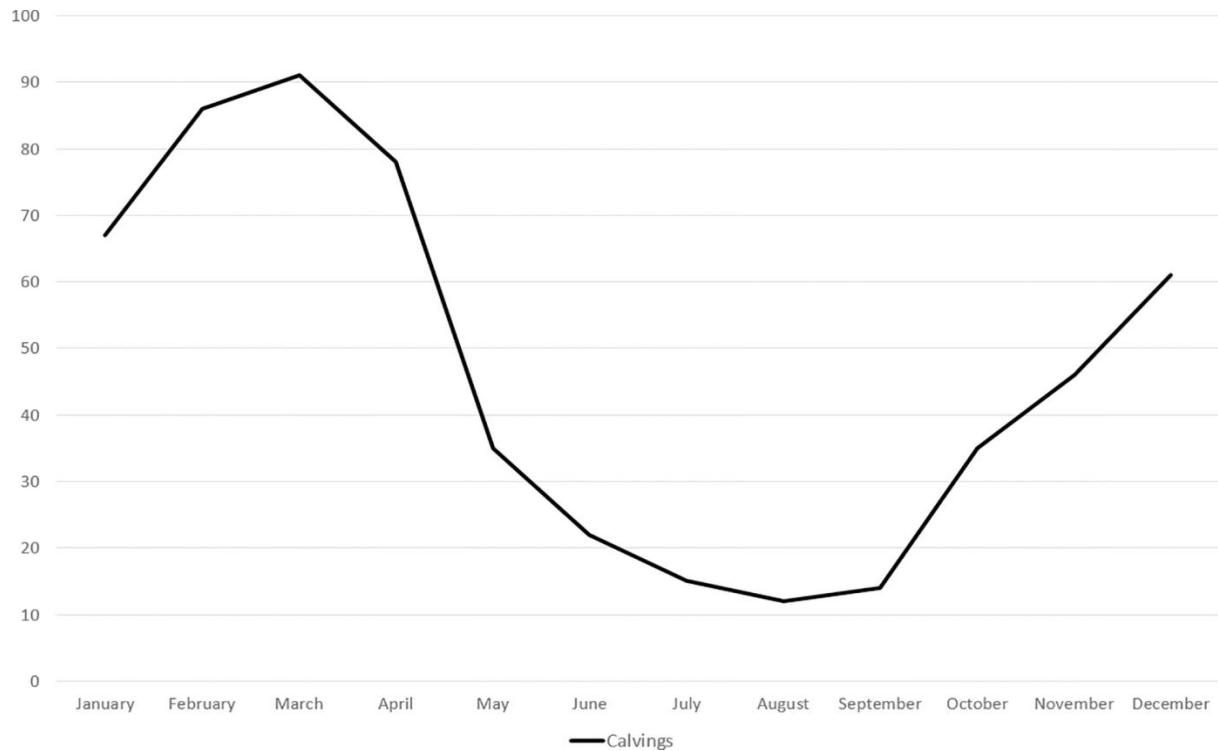


Figure 5. Season of calving. The line shows the total number of calvings throughout the study as divided per month of birth.

Effect of rainfall on number of births and sex ratios

Historic rainfall (mm/day) data was determined in the geographical area for 2008-2016 (Figure 6). Throughout the study period, based on the moment of conception, there was no significant effect of rainfall on the calf sex ratio.

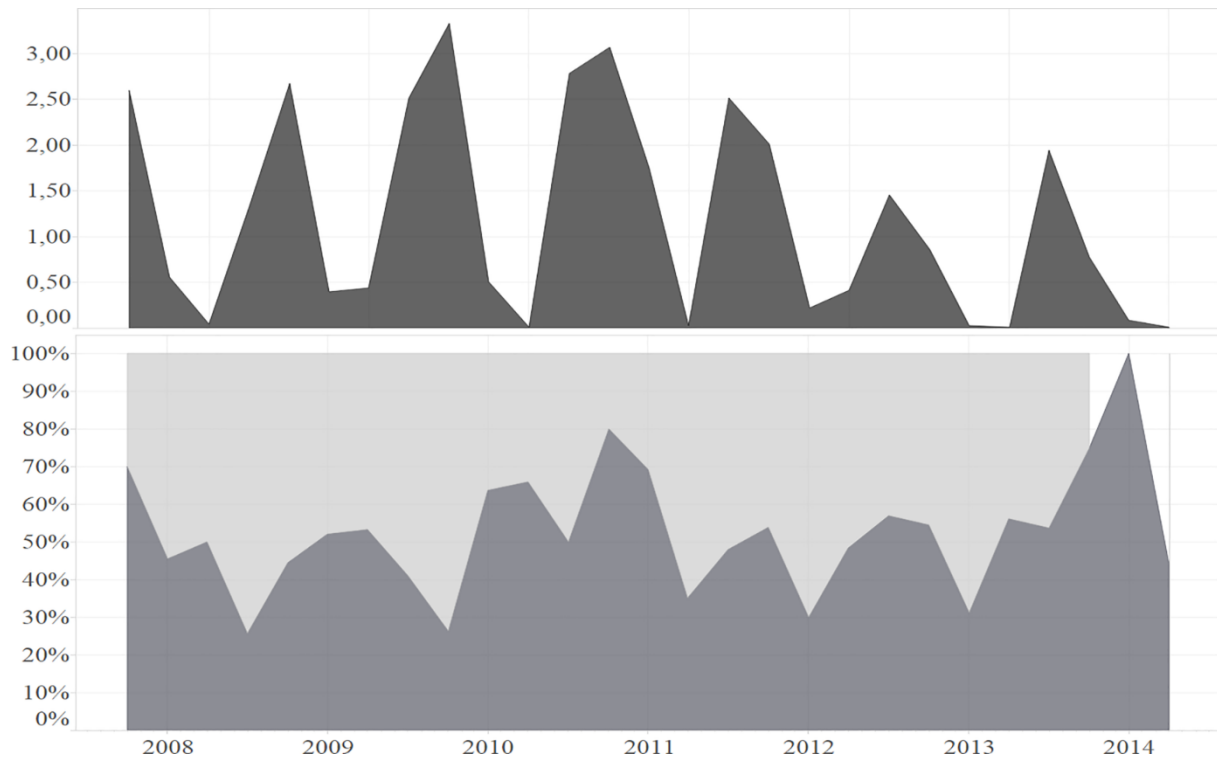


Figure 6. Daily rainfall (upper panel in mm) and percentage of male conceptions (lower panel in %) over time.

Discussion

In this study, we have analysed for the first time reproductive patterns in a 1,000+ population of game-ranched southern white rhinoceros. Captive breeding has become an important part of species conservation and offspring can be maintained as a genetic reservoir in case they should be needed for reintroduction into the wild or to supplement the existing population with new animals and new genetics (Magdalena Wolf et al. 1998; Fischer & Lindenmayer 2000). Wild southern white rhinoceros populations are decreasing dramatically due to induced death, with over 1,000 poached animals per year in the last three years (environmental affairs, RSA) (international 2016). The studied population contains more than 5% of the total population left worldwide and is larger than all southern white rhinoceros living in captivity worldwide (Foose & Wiese 2006). This game-ranched breeding institution creates new insights into management, reproduction, self-sustainability and animal welfare (based on the fact that healthy females usually breed well), and it represents a major resource for preservation of genetic diversity.

Reproductive behaviour and performance, as well as expression of appropriate social behaviour are two of the most common obstacles to conservation breeding. Therefore behavioural studies are needed (Lindburg & Fitch-Snyder 1994; Wielebnowski 1998; Swaisgood 2007). Decreased reproductive performance might be associated with the limited possibilities to exhibit normal reproductive behaviour patterns (Swaisgood et al. 2006). In these limited herd sizes from the global captive population, managed in very limited areas and sometimes totally different from the natural conditions of the rhino, the annual growth rate was negative (-3.5%) while growth rates in wild populations were still 6-10% (Emslie & Brooks 1999). Crucial to success in captive breeding systems seems to be an appropriate housing and management system, as well as correct diet.

We studied rhinoceros that were kept in large camps that contained at least one dominant male and several adult females. Polygamous breeding, where dominant males have access to several adult females seemed to be required (Owen-Smith 1975; Lindemann 1982; Fouraker et al. 1996). Looking at the key indicators in accordance with guidelines presented by the South African Development Community (SADC) Rhino program, one can conclude that this game-ranch facility had good to excellent reproductive performances (Du Toit 2006).

In our study, an average of 20% of the eligible females calved every year and 37% biennially (SADC classifies 33-40% as moderate to good) during the study period (7y). This number is a similar measure of performance to inter-calving interval, however the difference is that the average percentage of adult females calving per year also includes the eligible females that have not calved. Whether one should look at annual or biennial calving rate in animals with a gestation period of more than 12 months is open for discussion. Given the unique set-up of the studied facility, in which total herd growth or population growth was influenced by calves being born at the facility as well as by new animals that were introduced, it is difficult to compare these growth rates with those of previous studies done in wild or captive populations, in which introduction of new animals besides newborn calves is more rare. However, fig 1 showed that the artificial growth decreased during the study period and the calving rates increased. To avoid misinterpretation of the parameter population growth, we introduced the new parameter herd growth, since this study deals with game-ranch white rhinoceros. Herd growth of game-ranch white rhinoceros is

therefore the closest related parameter to compare with population growth in previous studies that looked at wild white rhinoceros. *Rachlow and Berger, 1998* (Rachlow & Berger 1998) showed a population growth between 6.6% and 10% in the wild. A population growth of 10.5% had previously been documented as a theoretical maximum for the species (Owen-Smith 1992). In our study the herd growth was on average 7%, which was mainly caused by the slow start of the breeding operation where population growth was mainly due to new arrivals. With a median age at first calving of 7 years of age (83.21 months), the breeding herd showed results in accordance with rapidly growing populations where females may have their first calves as young as at 6.5 years of age (Owen-Smith & Smith 1973). Other studies showed age at first calving to be 5.6-8 years (Patton et al. 1999) and 7.4 – 10.1 years (Rachlow & Berger 1998; Skinner et al. 2006). During the course of this study, the age at first calving did not increase significantly when numbers and density of animals increased. This is in contrast with observations in previous studies where a high-density population was compared to a low-density population (Rachlow & Berger 1998). The probable reason for age at first calving failing to increase as population density increased is the availability of feed.

Median inter-calving interval of 29.25 month (with < 30 months classified as good to excellent) is even an overestimation since some animals brought in with an incomplete calving history, exhibited a longer inter-calving interval. Animals with higher parity still showed regular inter-calving intervals of 25-27 months intervals, despite regularly anaesthetic procedures for management purposes. In previous studies free roaming rhinoceros showed a 30 months average inter-calving interval (Owen-Smith & Smith 1973), or even of 34.8 (low density) to 39.6 months (Rachlow & Berger 1998), whereas captive white rhinoceros showed a larger variation in inter-calving period from 19.5-29.5 months (91 births from 30 females)(Lindemann 1982) or 34 months out of 33 calving intervals calculated of different parity animals (Skinner et al. 2006).

In this study we also looked at translocation of animals when they arrived at the facility and the number of days till first calving at the facility. A slight drop in calving rates is seen in the first couple of months before and after arrival. This is probably because highly pregnant animals and freshly calved animals (calf on foot < 5 months) are very seldom translocated from one facility to another. Other than that the

translocation did not seem to affect the general fertility of the animals. However we did not have a control group of the same animals that calved without being translocated but only a follow-up in time of the same population. If we examine the survival analysis of the calvings of the animals that have been translocated into the population, we see the same slope in the first 60 months after calving. This shows that in a time period double the calving interval, there is no change in calving rate. In other words, fertility does not seem to be influenced by stress due to translocation. Even more, management manipulations that necessitate darting (anesthesia) performed on a regular basis, had no influences on fertility.

Our data showed a clear seasonal calving pattern with increased numbers from December until April. Most studies describe reproduction with seasonal peaks or as not seasonally restricted (Owen-Smith 1971; Skinner et al. 2006) but never with such a clear seasonal influence as shown in our data. In this study conception rates were higher during periods of supplemental feeding, therefore further research into nutritional influences of captive and wild diets on reproduction would be of great value. Previous studies pointed out that phytoestrogens might play a negative role in the reproductive success of captive white rhinoceros (Patisaul 2012; Tubbs et al. 2012; Patisaul 2013; Tubbs et al. 2016).

Out of 562 new-borns in the facility during the study, there was no significant skewed progeny sex ratio detected. The fact that the dam will adjust the sex of the offspring in response to environmental conditions in order to produce the male sex that has the greatest potential to reproduce, as described for the first time in the hypothesis of Trivers and Willard (1973), is currently observed in most captive breeding facilities (Trivers & Willard 1973; Garnier et al. 2001). This skewed sex ratio could ultimately result in further reducing the rhinoceros population reproductive success in future with bulls outnumbering cows. In this case, removal of males from small populations might be indicated for long term survival of the population. When we modelled the rainfall during the time of conception, then it showed no significant influence on the sex of the offspring, with an overall progeny sex ratio of 53.1% males.

Based on reproductive performances, we can conclude that it is possible to breed a large number of rhinoceros in game-ranched conditions that allowed exhibition of normal social behaviour and control of animal welfare by permanent veterinary

supervision and management of the habitat. Captive as well as wild reproductive performances were exceeded. Populations as studied might be of great genetic value for future genetic variability of free roaming populations and can help the fight against poaching. Poaching numbers almost exceed newborn numbers nowadays.

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CHAPTER 4

The effect of dehorning and other potential factors influencing horn growth in game-ranched white rhinoceroses (*Ceratotherium simum simum*)

Modified from:

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Abstract

Since 2008, poaching has put great pressure on the population of African rhinoceros. As such, dehorning of free-roaming and captive rhinoceros is regularly performed to discourage possible poachers. In this study, we analysed 2,044 dehorning events over a time span of 62 months from a 1,000+ herd of southern white rhinoceros (*Ceratotherium simum simum*) with a mean age at dehorning of 4.26 ± 2.3 yrs. To evaluate whether the growth of the horn was influenced by different factors, including age, the number of dehorning events and the seasonal timing of dehorning, a multivariate analysis was performed. The seasonal calving pattern observed in cows did not influence horn growth. In the total population, horn growth increased for each additional day of age (2.03 ± 0.066 g/day of age). Males showed a higher growth rate of horn (2.36 ± 0.082 g/day) compared to females (1.74 ± 0.073 g/day). Increased knowledge about horn growth in this species and about factors influencing this growth will contribute to general knowledge of game-ranched rhinoceros management and species conservation.

Introduction

The ongoing poaching of rhinoceros for use of the horn in traditional medicine has become a threat to the survival of all rhinoceros species. At present, every seven hours, a southern white rhinoceros (*Ceratotherium simum simum*) is poached in South Africa (Charlton 2017). Internationally, the trade in rhino horn was banned in 1977 by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). However, domestic trade continued within southern Africa's borders for three decades. In 2008, a sudden increase in rhinoceros poaching was observed, and the government of South Africa suspended the legalized domestic trade of horn, but the number of poached rhinoceros increased soon after. A year after this moratorium passed, South Africa lost 333 rhinoceros to poaching, followed by 448 in 2011, 668 in 2012, 1,004 in 2013, 1,215 in 2014, 1,175 in 2015 and 1,054 in 2016 (excluding the numbers of poached rhinoceros in Namibia, Botswana and Zimbabwe) (Charlton 2017). Rhinoceros horn currently has an estimated street value worth more than gold, diamonds or cocaine (Biggs et al. 2013). However, the horn is only keratin, without evidence-based beneficiary health effects (Laburn & Mitchell 1997). It can be compared to human fingernails, hair or equine hooves (Ryder 1962; Lynch et al. 1973; Sas-Rolfes 2012; van Hoven 2015). Trimming the back and front horn has been suggested as an effective anti-poaching measure in captive, game-ranched and free-roaming rhinoceros (Kagande & Musarurwa 2014; Actman 2017). Both the anterior and posterior horns are removed at a safe distance from the layer of dermal papillae, comparable with trimming the hooves of a horse, and the rhinoceros horn continues to grow naturally.

However, dehorning rhinoceros followed by stockpiling the horn with the aim to sell it has led to a passionate debate worldwide about the pros and cons of international legalization of the horn trade (Sas-Rolfes 2012; Collins et al. 2015; Crookes & Blignaut 2015; Di Minin et al. 2015; Taylor et al. 2017). Whatever the rationale, it is evident to all involved parties that rhinoceros conservation and the prevention of poaching in their natural habitat must be the central focus in this discussion. Trimming horns will not solve the poaching problem on its own, since rhinoceros have still been poached soon after their horns were trimmed. However, a reduction in poaching has been registered in reserves which apply regular dehorning (Kagande & Musarurwa 2014). Currently, creating value for live animals is a frequently used tool

in the protection of endangered species and has worked in many conservation efforts. Therefore, along with tourism, controlled trade in rhinoceros horn can become a sustainable source of income for the protection of rhinoceros (van Hoven 2015). Yet it has not been investigated whether legalizing the trade in rhinoceros horns has a dissuasive effect on the poaching of free-roaming rhinoceros species or whether leakage from the black market may potentially be fuelling further demand for poached horn (Di Minin et al. 2015; Taylor et al. 2017). Increased knowledge about horn growth in this species and factors influencing this growth will hopefully assist government and international agencies, wildlife reserves and other owners of rhinoceros in making management decisions about their dehorning procedures.

This study shares objective quantitative data for horn growth in combination with results from captive breeding and dehorning. In this retrospective study, we evaluated potential factors influencing horn growth in game-ranched white rhinoceros. We modelled factors like sex, age, calving, season and the number of dehornings as risk factors for horn growth and regrowth with respect to length, circumference and weight.

Materials and methods

Ethics statement

The authors confirm that all methods were carried out in accordance with relevant guidelines and regulations and that all necessary permits were obtained from the South African authorities before dehorning was carried out. All procedures were carried out as part of routine management and good veterinary practices. Retrospective data analysis was performed with the permission of the owner, and none of the animals were handled for the purposes of this research project.

Study site and animals

The study animals were game-ranched white rhinoceros from one facility in South Africa. The study contains data from 671 females and 395 males. The original data set used included records of 2,044 dehornings (1,297 from females and 747 from males). Records were taken between 23 October 2010 and 4 January 2016. Not every dehorning event comprised all measurements, due to time constraints under field conditions. All study animals appeared to be healthy prior to, during and after

the procedure. The rhinoceros were kept on an 8,000 hectare property divided into different breeding camps. In summer, the animals lived off natural field sources and additional mineral/vitamin licks. In winter, there was an additional supply of lucerne and pellets. The animals were dehorned on a regular basis (the median dehorning interval in this study was 587 days (IQR: 453–715)) as part of anti-poaching measures. Prior to the immobilization and the removal of the anterior and posterior horns of each rhinoceros, all necessary official permits were obtained from North West Nature Conservation. Representatives of the governmental authorities were also present at each dehorning to supervise the action and check the permits.

When an animal was identified from a distance by its ear notches, the size of the horns was estimated prior to administering the anaesthesia by a remote dart system. To minimize the number of immobilizations, dehorning was combined with other management or veterinary health interventions. Dehornings were performed throughout the year but only in cool and dry weather. Adult animals were darted in the neck, shoulder or semimembranosus/ semitendinosus musculature, with 4 mg of etorphine hydrochloride (Captivon, 9.8 mg/mL, Wildlife Pharmaceuticals, White River, South Africa) and 40 mg of azaperone (Stresnil, 40 mg/mL, Janssen Pharmaceutica, Johannesburg, South Africa); subadults with 2–3 mg of etorphine and 20–30 mg of azaperone; and calves >1 year with 0.5–2 mg of etorphine and 5–20 mg of azaperone. The dose of immobilisation cocktail used was adapted according to body size and condition of each individual. The complete dehorning procedure, from darting the animal until full recovery, took approximately 15 minutes. Photographs were taken before and after the horns were removed. The major curvature as well as the circumference of the bases of both the anterior and posterior horn were measured (Figure 1).

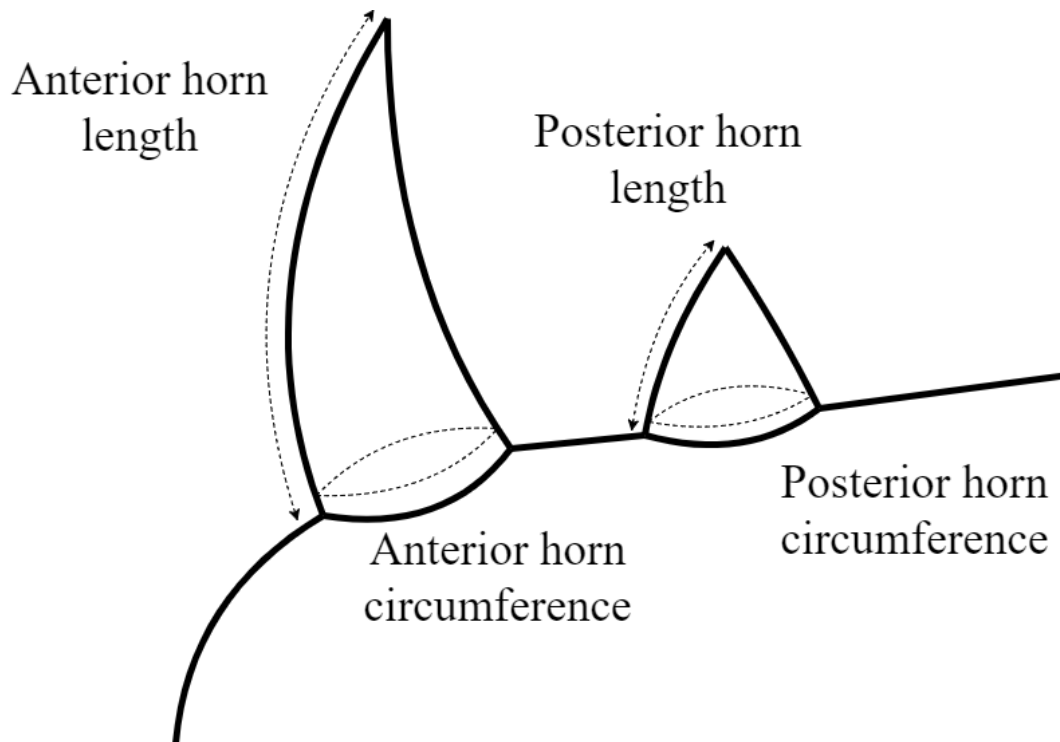


Figure 1. Example of a worksheet depicting how horn growth was measured.

During this conventional dehorning method, horns were trimmed at a safe distance from the horn growth plate (9–11cm) using an electric reciprocating saw (Makita® reciprocating saw). Once all procedures were completed, 20 mg of naltrexone (Trexonil, 50 mg/mL, Wildlife Pharmaceuticals) per 1 mg of etorphine was injected intravenously to reverse the opioids. During the rest of the day, treated rhinoceros were observed by rangers using binoculars.

Indicators of horn growth in the population of rhinoceros

In the study, the size of each individual anterior and posterior horn was measured. Both the anterior horn curve (length in mm) and the base circumference of the horn (length in mm) at the junction of the horn base and the skin were recorded (Fig.1). Eventually, the total weights (g) of the removed anterior and posterior horn as well as of the shavings were recorded. The growth of horn was defined relative to the previous dehorning event as a weight increase (grams per day), a length increase (mm per day) and a circumference (mm per day) for both the anterior and posterior horns.

Variables of interest which influence horn growth

The first model included factors influencing the total horn growth for females who calved at the facility during the period of dehorning. We took the overall growth of weight (per day) from the posterior and anterior horns together and analysed how this was influenced by the number of dehorning events in the study period, the age of the animals, the period between calving and the dehorning event, the parity of the females, the time of year in which the female calved and the time of year when the animal was dehorned. The number of dehornings varied from 1 to 5 ($n=381$). Calving dates and intervals were described in a previous study (Ververs et al. 2017).

In the second model, we looked at factors influencing the total growth of the horn (weight) of all animals with a registered date of birth. The median age of these animals was 4.25 years (IQR: 2.52–5.38). In this model, we could take the influence of sex into account with males ($n=588$) and females ($n=498$) as well as the influence of age, the number of dehorning events, the month of dehorning and the month of birth.

In a third, fourth and fifth model, we also used other models to look at such factors as sex, age, the number of dehornings, the time of the dehorning event and time of birth, influencing the anterior horn weight (third model), length (fourth model) and circumference (fifth model). Since the anterior horn is the largest, we assumed it had a major influence on total horn growth. For this reason, we used the anterior horn for this analysis.

Statistics

The statistical analysis was performed on different subsets of the original data. The descriptive statistics of all the dehorning data were calculated in R (Team 2017). The *summary* and *describe* function from the *base* and *psych* were used (Revelle 2017). Descriptive statistics (mean and standard deviation, or median and interquartile range) were reported for all horn measures grouped by sex unless explicitly stated for a subset of the data. Graphical data exploration was used to detect outliers and explore appropriate distribution and link functions for subsequent multivariate analyses. Generalised linear mixed models were constructed for each of the dependent variables using the *glm* function. The “log” link-function was selected

using the family = Gaussian (link = *log*) option. Univariate models were built for each of the independent variables, compared to the base model containing an intercept only. For the females only, the number of dehorning events, days since arrival, calculated number of calvings, days since last calving, dehorning quarter and calving quarter were tested. For the female and male model, the age, gender, number of dehorning events and quarter of dehorning were tested. Model comparison and final model selection was performed using the Chi-square test statistic from the deviance output using the ANOVA function from the *base* package. Biologically relevant interactions between each of the significant variables were tested and selected using the Chi-square test statistic from the deviance output using the ANOVA function and from visual exploration of the residuals. Next, the least square means (LSM) \pm standard error (SE) and *P*-values were constructed for the final model using the *lsmeans* function from the *lsmeans* package (Lenth 2016). To allow interpretation of the significant interaction terms, effect plots were constructed using the effect function from the effects package (Fox 2003).

To allow transparency in the data exploration and statistical analysis, the original dataset, R-markdown script and GitHub HTML document created using R-Studio (version 1.1.383, 2016) (Team 2016) were sent to an online repository publicly available at <https://github.com/Bovi-analytics/ververs-et-al.2018>.

Results

Descriptive statistics

For the total population, the median length of the posterior horn was only 34% of the median length of the anterior horn collected during dehorning. The median circumference of the posterior horn was 86% of the median circumference of the anterior horn. The median weight of the anterior horn was almost five times greater than the median weight of the posterior horn (Table 1). In total, 1.71 ± 0.98 (mean plus minus SD) kg of horn was removed per animal per year of the study.

Table 1. Descriptive statistics of the total study population. Horn measurements of the length and circumference were taken from the untrimmed horn and the weight of the horn from the trimmed horn.

Total (n=2044)	Mean	SD	Median	IQR
Age (days)				
- Overall	1554.74	839.96	1379	924 - 1967
- Animals dehorned one time	1233.80	796.19	966	760 - 1415
- Animals dehorned two times	1766.76	749.66	15542	1340 - 1868
- Animals dehorned three times	2262.73	528.37	164	1901 - 2494
Anterior horn length (mm)	168.81	91.64	145	119 - 185
Anterior horn weight (g)	1338.94	738.70	1263	849 - 1670
Anterior horn circumference (mm)	572.67	108.74	590	505 - 645
Posterior horn length (mm)	56.60	30.79	50	40 - 65
Posterior horn weight (g)	308.93	246.84	259	142 - 406
Posterior horn circumference (mm)	491.24	127.77	510	420 - 575
Shavings weight (g)	103.89	57.64	99	61 - 104
Regrowth anterior horn in length (mm/day)	0.25	0.58	0.22	0.17 - 0.26
Regrowth anterior horn in weight (g/day)	2.39	8.69	1.71	0.81 - 2.5
Regrowth posterior horn in length (mm/day)	0.09	0.31	0.07	0.05 - 0.09
Regrowth posterior horn in weight (g/day)	0.62	2.86	0.32	0.11 - 0.55
Interval between dehorning events (d)	589.90	206.20	587	453 - 715

SD: standard deviation, IQR: interquartile range

The study animals were part of a large breeding facility with 37% males and 63% females. Age was not significantly different between the males (mean $1,576 \pm 812$ days) or females (mean $1,529 \pm 871$ days) in the study. Interval between dehornings, once horn growth was acceptable (estimated by visual observation), was determined on availability of government permits and planning.

At the moment of the dehorning event, 74% of the females were pregnant, and 100% of these calved successfully without any complications. For animals in which the subsequent calving date was recorded ($n=142$), we found that the median number of days from dehorning till calving was 278 (IQR: 161–495).

Factors influencing total growth (weight; g/day) of horn in reproducing females

The timing of calving, which showed a clear seasonal pattern (Jan–Mar: $n=69$, Apr–Jun: $n=39$, Jul–Sep: $n=35$ and Oct–Dec: $n=18$), did not exert any influence on horn growth. We looked at 142 distinct individual females. The period between calving and the dehorning event did not affect the total growth of the horn either.

Out of these four seasonal groups (Jan–Mar, Apr–Jun, Jul–Sep and Oct–Dec), we noted a higher growth ($P=1.50 \cdot 10^{-3}$) of horns in females which had been dehorned ≥ 3 times (5.05 ± 0.53 g/day) ($n=24$; age: IQR: 1333–1847 days) when compared to females which had been dehorned 2 times (2.74 ± 0.41 g/day) ($n=135$; age: IQR: 913–1641 days).

In evaluating the effect of the timing of dehorning throughout the year on horn growth, we measured an increase ($P=4.41 \cdot 10^{-2}$) of growth from animals dehorned in the second quarter (Apr–Jun) (3.03 ± 0.45 g/day; $n=44$) compared to the first quarter of the year (Jan–March) (4.92 ± 0.68 g/day; $n=18$).

Factors influencing total growth of horn (weight; g/day) in males and females

We noticed a difference ($P < 2.20 \cdot 10^{-16}$) in growth of the total weight of the horns when the studied animals were divided per the number of dehorning events (1: $n=597$; 2: $n=315$; ≥ 3 : $n=174$). The least square means for the groups were 0.85 ± 0.06 g/day for those dehorned once, 2.91 ± 0.08 g/day for those dehorned twice and 3.58 ± 0.12 g/day for animals which had been dehorned three times or more. At least part of the effect was likely due to the effect of age.

A sex influence was also noticed, with a growth difference in weight ($P=2.34 \times 10^{-15}$) between males (2.41 ± 0.08 g/day; $n=588$) and females (1.77 ± 0.07 g/day; $n=498$).

Factors influencing growth of anterior horn weight (g/day) in males and females

We noticed a difference ($P < 2.20 \times 10^{-16}$) in growth of the anterior horn weight when the studied animals were divided per the number of dehorning events (1: $n=595$; 2: $n=314$; ≥ 3 : $n=174$). The least square means for the groups were 0.709 ± 0.049 g/day for those dehorned once, 2.33 ± 0.06 g/day for those dehorned twice and 2.72 ± 0.12 g/day for animals which had been dehorned three times or more. Part of the effect was likely due to the effect of age.

A sex difference was recorded with a growth difference ($P=6.05 \times 10^{-13}$) between males (1.86 ± 0.06 g/day; $n=588$) and females (1.46 ± 0.06 g/day; $n=495$). A considerable interaction ($P=5.69 \times 10^{-2}$) was found between the number of dehornings and age. The interaction is illustrated in the plot shown in figure 2. A subadditive effect for the growth of the anterior horn weight was observed in dehorning 1 versus dehornings 2 and 3 (Figure 2).

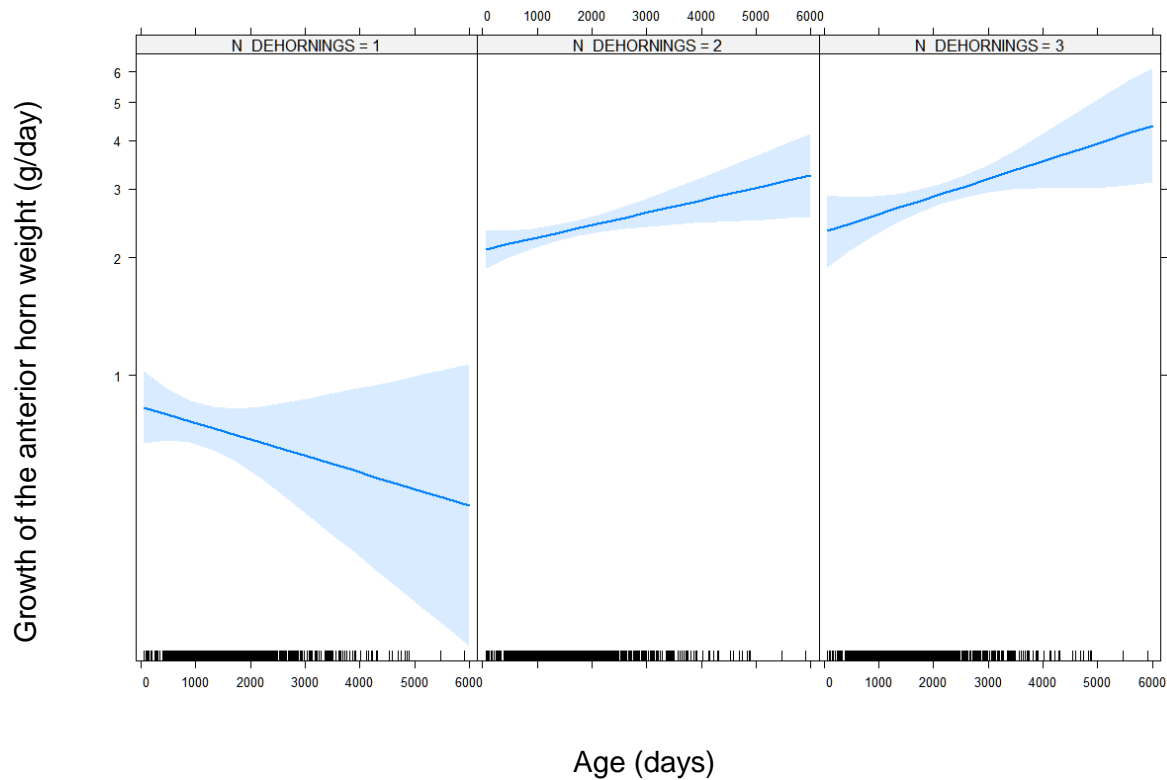


Figure 2. The effect plot shows the least square means to illustrate the interaction between the number of dehorning events (*top*) and the age of the animal (*x-axis*; days) for the growth of the anterior horn in weight (*y-axis*; g/day). The blue zone shows the confidence interval. A non-linear y-axis was used to increase interpretability.

Factors influencing growth of anterior horn length (mm/day) in males and females

We noticed a difference ($P=2.20 \cdot 10^{-16}$) in growth of the anterior horn length when the study animals were divided per the number of dehorning events (1: $n=590$; 2: $n=314$; ≥ 3 : $n=174$). The least square means for the groups were 0.14 ± 0.01 mm/day for those dehorned once, 0.29 ± 0.01 mm/day for those dehorned twice and 0.30 ± 0.01 mm/day for animals which had been dehorned three times or more. Part of the effect was likely due to the effect of age, because for each additional day of age.

A sex influence was noticed with a growth difference ($P=4.63 \cdot 10^{-2}$) between males (0.24 ± 0.01 mm/day; $n=584$) and females (0.23 ± 0.01 mm/day; $n=494$).

A significant interaction ($P=7.26 \times 10^{-12}$) between the number of dehornings and age was found. The interaction is illustrated in a plot shown in figure 3.

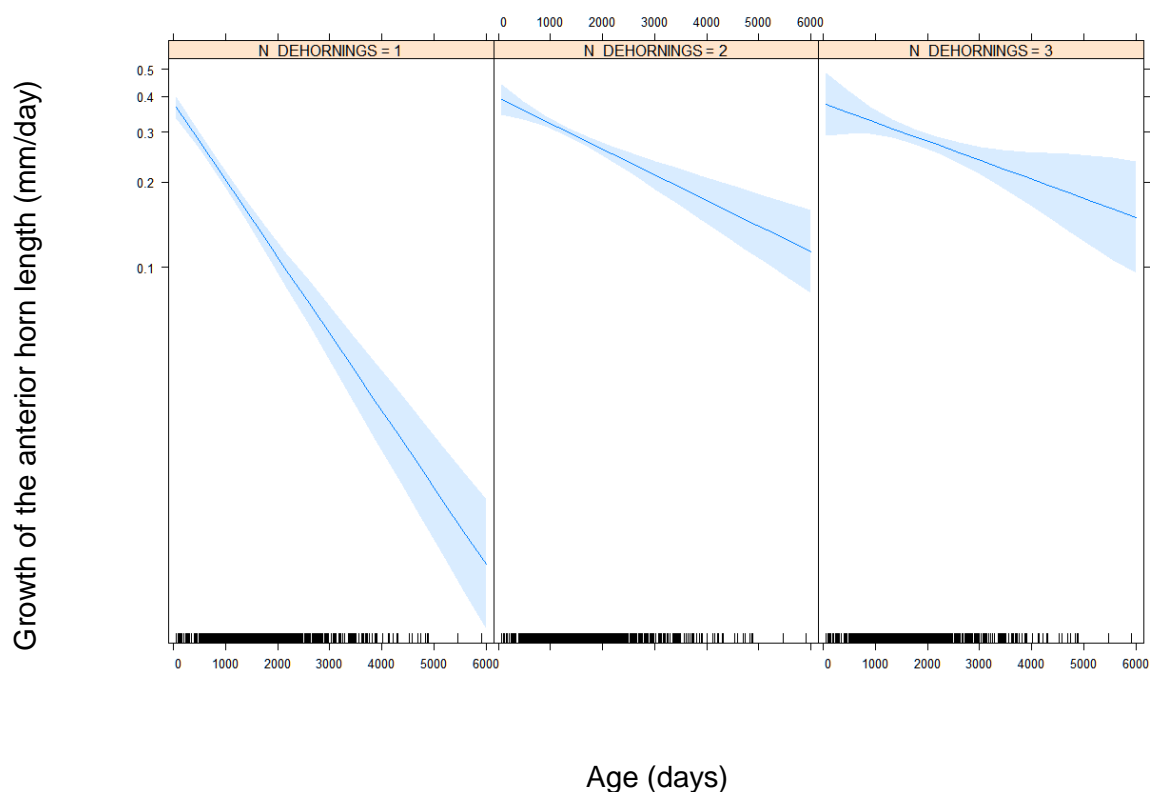


Figure 3. The effect plot shows the least square means to illustrate the interaction between the number of dehorning events (*top*) and the age of the animal (*x-axis*; days) for the growth of the anterior horn length (*y-axis*; mm/day). The blue zone shows the confidence interval. A non-linear y-axis was used to increase interpretability.

From figure 3, a stronger subadditive effect for the growth of the anterior horn length was observed in dehorning 1 when compared to dehornings 2 and 3.

Factors influencing the growth of the anterior horn circumference (mm/day) in males and females

We noticed a difference ($P=2.20 \times 10^{-16}$) in growth of the anterior horn circumference when the studied animals were divided per the number of dehorning events (1: $n=573$; 2: $n=314$; ≥ 3 : $n=173$). The least square means for the groups were 0.31 ± 0.03 mm/day for those dehorned once, 1.16 ± 0.03 mm/day for those dehorned twice and 1.22 ± 0.07 mm/day for animals which had been dehorned three times or more. At least part of the effect could be explained by the effect of age.

A sex influence was noticed with a growth difference ($P=4.05 \cdot 10^{-5}$) between males (0.81 ± 0.03 mm/day; $n=572$) and females (0.71 ± 0.03 mm/day; $n=488$).

An interaction ($P=9.64 \cdot 10^{-10}$) between the number of dehorning events and age was found. The interaction is illustrated in a plot shown in figure 4.

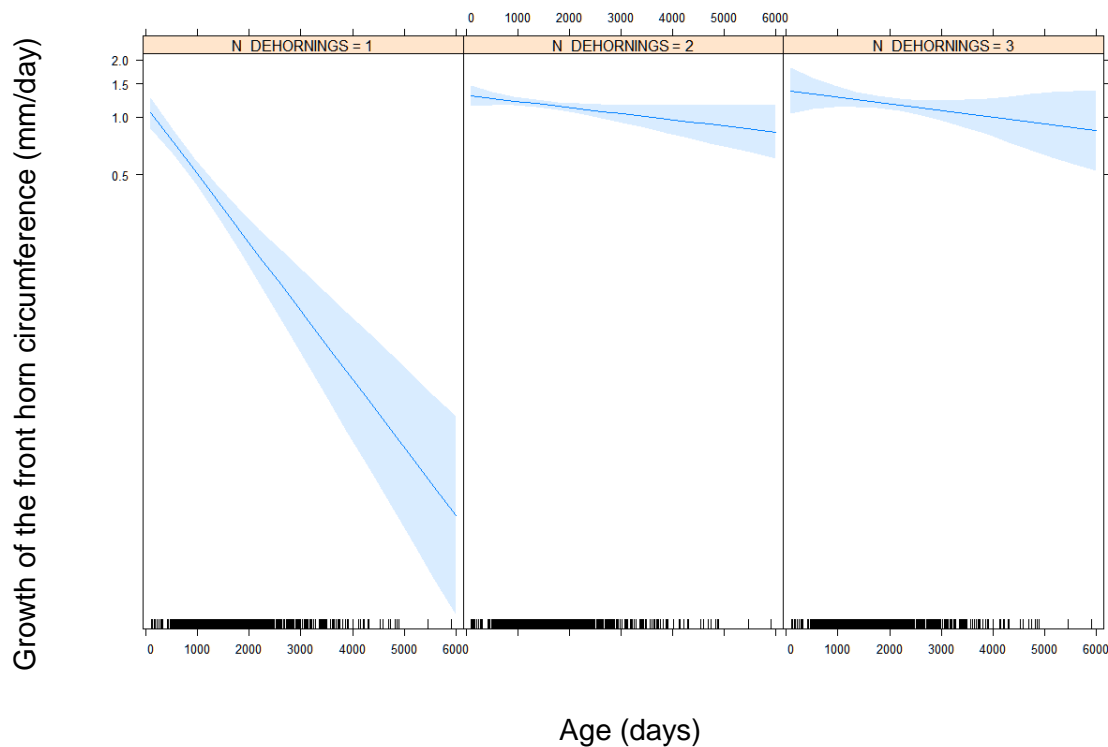


Figure 4. The effect plot shows the least square means to illustrate the interaction between the number of dehorning events (*top*) and the age of the animal (*x-axis*; days) for the growth of the anterior horn circumference (*y-axis*; mm/day). The blue zone shows the confidence interval. A non-linear y-axis was used to increase interpretability.

From figure 4, a stronger subadditive effect for the growth of the circumference of the anterior horn was observed in dehorning 1 when compared to dehornings 2 and 3.

Discussion

In this study about the horn growth in white rhinoceros ($n=2044$), we recorded a median intrinsic growth of 79 mm (IQR: 62–95) per year (mean: 0.25 mm/day). The median growth of the anterior horn was 73 mm (IQR: 59–91) per year (mean: 0.26 mm/day) for the females ($n=1297$) and 85 mm (IQR: 71–98) per year (mean: 0.24 mm/day) for the males ($n=747$). The higher ages of the animals with more dehorning events likely contributed to the significant differences ($P<0.01$) in growth of the anterior and posterior horn length in relation to the number of dehorning events. Consequently, based on these findings, it was not possible to conclude that the age at the first dehorning or the number of dehorning events directly influenced the horn growth. Due to the conical structure of the rhinoceros horn, most of the weight is localized at its base. Up to the first dehorning, the horn has time to grow in its natural conical shape, and thus, with increasing time, there will be less weight and circumference near the tip of the horn as well as less length, due to rubbing and breaking of the horn. It also appears that the growth rate of horn accelerates in the first year after horn loss (Pienaar et al. 1991). The super-additive effect of the front horn weight growth from animals dehorned more than once, with a subadditive effect for length and circumference, indicates that the total weight is influenced by more than just the length and the circumference (weight cone = $1/3\pi r^2 h \cdot \text{density}$). Perhaps the density of the keratin at the base of the horn is greater than at the tip. This is also in accordance with what Boy *et al.* (2015) found when they described how with increased length, columns of keratin undergo dehydration and shrinking. Therefore the weight increase may be greater if dehorning is performed at shorter intervals. During this period, the base of the horn grows and contributes more to the weight increase. Because of anti-poaching measures, horn is only given a minimal chance to regrow before a second dehorning. During this period, it is mainly the horn stump which regrows, with most of the weight onset in it.

The growth rates of the anterior horn in length (0.17–0.26 mm per day) were comparable with the growth rates of the equine hoof (0.14–0.48 mm per day) (Reilly et al. 1998) and bovine claws (0.13–0.24 mm per day) (Vermunt & Greenough 1995), but the growth rate of the posterior horn in length is much lower (0.05–0.09 mm per day). However, the growth rates found in this study were higher than those described in previous literature on the growth of rhinoceros horn (Kloes 1969; Pienaar et al.

1991). For example, an average of 50.50 ± 3.52 mm per year (0.14 mm per day) for anterior horn growth was found by measuring the movement of a microchip from the horn base per year, in wild adult white rhinoceros ($n=6$) (Pienaar et al. 1991). When dehorning was conducted on white rhinoceros in Zimbabwe, the average growth of the anterior horn was 67 mm per year (0.18 mm per day) and for the posterior horn ($n=14$) was 27 mm per year (0.07 mm per day) (Kock and Atkinson, 1993). In another study, horn growth rates were measured by tape measure and without dehorning in a captive male and several female rhinoceros ($n=2$). The horn of females of 5–8 years showed an annual growth rate of 58.50 ± 8.16 mm (0.16 mm per day) and that of the male a growth rate of 47.80 ± 5.80 mm (0.13 mm per day) (Kloes 1969). Our study animals received additional high-quality feed during the dry season, which likely had a positive effect on growth compared to free-roaming individuals, since the latter often show a severe decrease in body condition during dry periods. Assumptions on the growth influence of the diet have been made in a study on the horn composition of the ancient woolly rhinoceros (Tiunov and Kirilova, 2010). Additionally, in free-roaming conditions, more energy is likely needed for grazing, traveling larger distances and interacting with other species. In cattle, it has been suggested that hormones, vitamins, minerals and trace elements (for example sulphur-containing acids, biotin and calcium) play critical roles in the normal development of horn and in the formation of keratin in the claw. In early lactating dairy cows, compromised production of claw-horn keratin was detected when there was a decrease in insulin sensitivity and/or concentration, which may be related to the binding of insulin to both the epidermal and dermal layers of explanted bovine hoof tissue, and a deficit may lead to lower glucose availability for tissue growth (Bragulla et al. 1999; Hendry et al. 1999; Tomlinson et al. 2004). Although nutrition was not taken into account in our study and is outside the scope of this paper, the authors suggest that nutrition likely has an influence on the growth of horn, and it will be worthwhile to include this factor in future studies on horn growth in captive rhinoceros.

A recent study by Boy *et al.* (2015) provided new insights into the macroscopic and microscopic structure of rhinoceros horn and showed that horn development at the base includes some underlying dermal connective tissue, a dermal papillae layer (15 mm in length) and a horn structure on top (13 mm). The dermal papillae layer is covered by onychokeratinizing epithelium, which gives origin to the corneocyte

columns responsible for the entire horn structure. Due to the different long-axis orientations of these corneocytes, the columns are tightly packed with no hollow tubules or cavities. The corneocyte columns which are associated with each dermal papilla elongate individually and fuse with the central part of the horn. The horn base increases in diameter with advancing age by adding more dermal papillae at the periphery of the lengthening mass of corneocyte columns. These new peripheral columns fuse with the central part and are shorter because of their more recent development. As the horn increases in length, the most distal columns undergo dehydration and shrinking. This enhances the conical form of the rhinoceros horn, which might already have been initiated, with the slanting seen in the early developing calf's horn (Boy et al. 2015). There are different dehorning techniques but the conventional (with a stump of horn left) and the Kock and Morkel (with the complete horn removed and trimmed all around to the base) methods are the most popular ones employed. In this study, the conventional method was used. When the Kock and Morkel method is applied, horn measurements will probably be different, as the total horn mass removed during dehorning is larger. Horn sizes and growth can also be measured based upon a microchip placed in situ and leaving the horn on the animal (Pienaar et al. 1991) or by measuring the horn when removed for management purposes, as presented in this study.

It has been stated that horn regenerates about 8.7 cm per animal per year (2.38 mm/day) and that the regrowth of horn weight exceeds 500 g per year (1.37 g/day) (Berger & Cunningham 1994). Pienaar *et al.* (1991) showed that the annual horn growth of anterior horns appears to decrease with age, with intrinsic growth being higher in young adults than in old adults. They showed an intrinsic horn growth of 59.8 ± 4.31 mm per year (0.16 mm/day) ($n=6$) for young adults (8–25 years) and 36.5 ± 9.04 mm per year (0.1 mm/day) ($n=4$) for old adults (>25 years). In our study, we examined the influence of age on the total weight growth of the anterior and posterior horns and on the growth of anterior horn length, weight and circumference. Only the growth of the anterior horn weight increased with age when animals were dehorned two times or more. For length and circumference there was less growth increase recorded with increasing age of the animals. The mean age of our study animals was 4.25 years (IQR: 2.52–5.38), which is still very young. The growth curve of the horn might therefore change when the animals get older.

Average regrowth rates for adults were not found to differ significantly between the sexes (Kock & Atkinson 1993). In another study, it was said that there was only sexual dimorphism in the growth of the horn, in the ratio of anterior-to-posterior horn length, with females having a slightly longer anterior horn than males, and no sexual difference could be found between the total masses of the anterior and posterior horns (Milner-Gulland et al. 1992). Previous researchers found that the anterior horn base as well as the total horn weight of the anterior and posterior horns was greater in males compared to females, while others stated that adult males produced almost twice the mass of horn annually as adult females of similar ages (Pienaar et al. 1991; Rachlow & Berger 1997). In the present study, the anterior-to-posterior horn ratio was slightly higher in males (3) than in females (2.6). The age range was 58–5,468 days in males and 94–5,909 days in females. Here, the growth of the total weight of both horns ($P < 0.01$) as well as the anterior horn weight ($P < 0.01$), the anterior horn length ($P < 0.05$) and the anterior horn circumference ($P < 0.01$) were higher in males than in females. The mean horn circumferences measured during each dehorning were bigger in the anterior (580 ± 125 mm) and the posterior ($519 \text{ mm} \pm 144$) horns of the males when compared to the anterior (568 ± 98 mm) and the posterior (475 ± 114 mm) horn circumferences of the females. Other studies showed circumferences of 702 ± 15.8 mm (anterior horn) and 582 ± 18.7 mm (posterior horn) in males and 609 ± 9.0 mm (anterior horn) and 493 ± 9.5 mm (posterior horn) for females (Rachlow & Berger 1997). Horn regrowth in this latter study was similar to that in our results. Rachlow and Berger (1997) found that females reached a peak in horn regeneration at 8 years, whereas the mass of horn regenerated by males approached an asymptote slowly at over 30 years of age. Our study mainly collected data from animals of a younger age.

Dehorning plays a significant role in rhino conservation by reducing poaching. (Kaganda & Musarurwa 2014; Taylor et al. 2017). However, major considerations should be made when implementing dehorning strategies in conservation of the white rhinoceros. First of all, the positive and potential negative effects of horn trimming and anti-poaching measures should be evaluated. Their ability to survive, display their natural behaviour and protect their offspring without horn should be evaluated (Berger & Cunningham 1994; Lindeque & Erb 1995; Berger & Cunningham 1996). Previous research showed that white rhinoceros bulls are minimally territorial and

only occasionally use their horn in stand offs against other bulls to defend their territory (Owen-Smith 1971). Badenhorst et al. 2016 studied stress steroid levels and the short-term impact of routine dehorning. A short-term stress response showed a significant increase in faecal glucocorticoid metabolite levels 48h post-dehorning (regardless of previous dehorning experience), that returned back to pre-dehorning concentrations 72h after the procedure. In contrast, increased faecal glucocorticoid metabolite levels have been reported for longer than 75 days in female white rhinos following immobilisation and relocation (Linklater et al. 2010).

An integrated and holistic approach is required to effectively protect the rhinoceros (Kagande & Musarurwa 2014). In the authors' opinion, the long-term survival of the species must be the main focus of all people, researchers, conservationists and private owners involved. The solution to rhinoceros poaching most likely comes from a combination of all anti-poaching measures, including demand reduction, end-user education and a controlled and traceable supply from game-ranched rhinoceros.

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CHAPTER 5

Faecal androgen levels in different age classes and their relation to the breeding status in game-ranched male southern white rhinoceroses (*Ceratotherium simum simum*)

Modified from:

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Abstract

Androgens are involved in the onset of puberty and sexual behaviour in mammals. In wild animals like rhinoceros, faecal androgen metabolites may be used as a tool to determine whether a male has reached puberty, and to identify breeding bulls. The aims of this study were to investigate the effects of age, social structure and breeding activity on faecal androgen metabolite concentrations (fAM) of southern white rhinoceros bulls. The study animals ($n = 69$) were part of a large group ($n = 1,354$) of game-ranched male southern white rhinoceroses (*Ceratotherium simum simum*) in South Africa. Faecal samples were collected and analysed for immunoreactive androgen metabolites. Median fAM-levels in faecal samples of adult males (298 (127-829) ng/g dry weight) were higher ($P < 0.001$) than in the samples of subadults (153 (108-336) ng/g dry weight) and juveniles (156 (119-227) ng/g dry weight). A difference ($P = 0.04$) in median fAM levels was found in subadults that already expressed breeding behaviour (178 (152-336) ng/g dry weight) in comparison to subadults that did not express breeding behaviour (148 (108-250) ng/g dry weight). The presence of adult females seemed to play a role in the onset of sexual maturity of subadults, although no significant differences were observed.

Introduction

White rhinoceroses (*Ceratotherium simum*) are under serious threat, mainly due to increased numbers of man induced deaths, caused by poaching and habitat loss. Numbers of living white rhinoceros are currently below 20,000 worldwide (Amin et al. 2006; Emslie R. 2017). An important part of saving the species from extinction, is obtaining knowledge about their reproductive physiology. Breeding attempts in captive settings have so far not been very successful (Swaisgood et al. 2006). Most studies tend to focus on female reproductive physiology, whereas the male side is often neglected (Roth 2006; Roth et al. 2017). In captive female rhinoceros, the oestrous cycle appears to be irregular, with either a short fertile or a longer infertile cycle (Radcliffe et al. 1997; Hermes et al. 2004; Hermes et al. 2006). In free roaming females the oestrous cycle appeared to be rather regular with about 30 days interval (Schwarzenberger et al. 1998; Goot et al. 2013). Both females and males, mainly in captivity, often suffer from reproductive pathologies (Hermes et al. 2005; Portas et al. 2005; Hermes et al. 2006).

Good reproductive performance in a population is heavily influenced by nutritional and environmental factors (Metrione et al. 2007; Tubbs et al. 2016). Additionally, social structures and interactions between and within sexes are of great importance. The social group structures in the wild will be that adult females, subadults and juveniles live in overlapping home ranges, mostly in groups of two to three individuals. Sub-adulthood (Puberty) commences when the calf is expelled from the social group by its mother and ends when social-sexual maturity is attained. This occurs at two to six years of age. In captivity sexual maturity tends to occur earlier, with females becoming sexual mature earlier than males. Subadult white rhinoceros create cohesive pairs or larger groups involving subadults only or subadults with adult females without a calf on foot (Owen-Smith & Smith 1973; Owen-Smith 1975; Owen-Smith 1992; Shrader & Owen-Smith 2002). Adult southern white rhinoceros males are solitary and defend their territories, which are exclusive of other males (Owen-Smith 1971; Rachlow et al. 1999). Factors such as social ranking within a population, size of the population and dominance but also sensory stimuli from the opposite sex, impact on their sexual behaviour and reproductive performance (Metrione & Harder 2011). Onset of puberty, increase of sex hormone production, onset of sexual maturity and capability to reproduce can all be influenced by

environmental and social stimuli (Christensen et al. 2009; D'Souza-Anjo et al. 2017). Male rhinoceros maturity does not only depend on age, but is definitely related to many other stimuli. This could be one of the reasons why reproductive failure is frequently observed in captive herds, while wild populations show good reproductive performance. Captive-bred bulls that were unsuccessful in captivity were able to breed successfully following release into the wild (Swaigood et al. 2006; Swaigood & Schulte 2010). Otherwise, dominant white rhinoceros breeding bulls that have sired calves and taken out of their herd and reintroduced in another herd may fail to reproduce ever again. Males often need other males in their surroundings to determine dominance and to mature sexually. Presence of females, whether or not in heat, can have a significant impact on the onset of sexual maturity and development of sexual behaviour (Kretzschmar et al. 2004).

Faecal androgen metabolite concentrations (fAMC) are indicators for Leydig cell activity in male rhinoceroses. A previous study developed and validated an Enzyme Immunoassay (EIA) to identify two faecal metabolites similar to testosterone and dihydrotestosterone (Kretzschmar et al. 2004). They performed a longitudinal study on 5 individuals, looking at breeding status and seasonal influences. Androgen metabolite concentrations increased with sexual maturation and there was a correlation between plasma testosterone concentration and faecal androgen metabolite concentrations (Kretzschmar et al. 2004). Black rhinoceros that were successful breeders had significantly higher fAMC than males that had not bred. There was also a positive correlation between average testosterone levels and the number of offspring sired per year spent in the reproductive age class (Edwards et al. 2015).

In polygynous species, as is the rhinoceros, reproductive skew among males is common, meaning that only a small number of males is responsible for a high proportion of the matings (Clutton-Brock 1989; Garnier et al. 2001).

This study investigated the effects of herd structure, age and reproductive performance on fAMC of game-ranched white rhinoceros bulls. Our hypothesis was that males in breeding groups would exhibit higher faecal androgen metabolite concentrations than males from non-breeding groups and that there would be a positive correlation between age and faecal androgen metabolite concentrations.

Breeding bulls are bulls that have sired calves (proven sires) and non-breeding bulls have not produced progeny.

Materials and methods

Study area and animals

The study animals (n = 69) were part of a confined private game-ranched southern white rhinoceros population (n=1,354) and were kept in large breeding camps or male-only camps (9 ha per adult animal). The study was non-invasive and was conducted with consent of the owner. The game-ranch was located in a semi-arid climate, with high summer temperatures and short, cool, dry winters with frost. The mean annual precipitation was 530 mm, with most rainfall occurring during the summer months. Average midday temperatures for the area ranged from 18°C in June to 29.5°C in January. The region is the coldest during July when the temperature drops to 0°C on average during the night. Access to drinking water was ensured throughout the year. The facility consists of several adjoining properties divided in different breeding camps with 25-70 animals per camp depending on its size and location. To prevent overcrowding of camps, subadults (31-71 months) were removed from natal camps and placed in new camps to establish new breeding herds. This allowed population control and prevented inbreeding. In addition, new arrival adult bulls were introduced to the herds from outside the facility. Each breeding camp contained 1-3 adult breeding bulls with 25-30 adult females, 10-25 calves and 10-25 subadults. Isolated male camps were separated from breeding camps by the minimum of approximately 10 meter and the maximum as big as the camp sizes (which is different per camp), so olfactory and visual stimuli might still be possible.

Each breeding camp was divided in two sections to allow rotational grazing over the summer months, and rhinos were given additional feed when natural grass was limited. Supplementary feed consisted of alfalfa (8 kg) and pellets (5 kg) per rhino daily during the dry winter season (June-August), then in decreasing amounts until adequate rainfall allowed the return to a fully natural grazing diet. Pellets are partially manufactured at the facility. They contain a mix of sunflower oilcake, hominy chops,

feedlime, molasses syrup, alfalfa, oats, etc. Additional feeding during the winter was designed to maintain a good body condition of the animals and assure their general well-being. Additional husbandry requirements, including adequate shelter, shade, mud baths and rubbing posts were also available within each campsite. Natural mating occurred without human intervention other than controlling the breeding camp numbers and monitoring the carrying capacity of the field and camp with the minimal surface per rhinoceros set at 9 ha per animal. The rhinoceros were allowed to create their own natural hierarchy and herd structures. An ID-microchip (Identipet) was applied in the left side of the neck of each rhinoceros, and notches were applied in both ears for identification from a distance. A permanent full-time veterinarian was present on the premises to carry out preventative medicine and veterinary intervention when needed.

Observed mounting behaviour was recorded during roll calls three times a day. Oestrous females showed obvious signs of mounting such as scuff marks on their flanks and back from the legs of the bull. The identity of the bull and the cow was recorded to monitor the fertility of each bull and to predict the possible parturition dates for the cow. At each calving, the sex of the calf and the identity of the cow was recorded either early in the morning or late in the afternoon.

Males were classified as juvenile (21-30 months), subadult (31-71 months) and adult (72 months and older). Mating behaviour was registered and if successful, calving and resulting offspring was registered about 16 months after mating.

Collection of faecal samples

One faecal sample of each study animal ($n = 69$) was collected. The collection period was February 2014 ($n = 5$) and October until December 2014 ($n = 64$). Samples were obtained directly from the rectum during anaesthesia for management purposes ($n = 60$) or during observation sessions when defecation was seen ($n = 9$). Approximately 50 g of homogenized fresh faeces (mixed by hand) was collected and cooled immediately in a cooler box (5°C) after collection and frozen within 4 hours of collection at -20°C in faecal sample jars with screw-top lids. They were transported to the Endocrine Research Laboratory, University of Pretoria for processing and analysis, in a cooler box with frozen icepacks.

Sample storage and analysis

The frozen faecal samples were freeze-dried and subsequently pulverized. 100-110 mg of faecal powder (the exact weight was recorded and used for calculation) was used for steroid extraction in 3 ml 80% ethanol in water, by mixing for 15 min on a multi-tube vortex, and subsequent centrifugation for 10 min at 1500 x g. The supernatant was decanted into labelled micro-titer tubes, and stored at -20°C until analysis. An Enzyme immunoassay (EIA) was used to determine the levels of faecal androgen metabolites (fAM) similar to testosterone. The antibody was raised against testosterone-3-CMO:BSA. The label is 5 α -androstane-3 β , 17 β -diol-3-HS:DADOO-biotin and the standard is testosterone (Palme and Möstl 1993). The coefficients of variance for intra-assay-variance are 4.7% - 6.2% and the coefficients of variance for inter-assay-variance are 9.1% - 10.2%. The sensitivity of the EIA used is 2.4 ng/g dry faeces (T-3-CMO).

Data analysis

Faecal androgen metabolite (fAM) concentrations were compared between the three defined age groups (juveniles, subadults and adults) by non-parametric data analysis taking into account the lack of homoscedasticity of the data (Kruskal-Wallis Test with Post Hoc Dunn's multiple comparison test).

Additionally, the influence of the presence or absence of (mature) females in the group on the hormone levels, as well as the difference in hormone concentrations between bulls exhibiting breeding behaviour and non-breeding behaviour was investigated, in both the subadult and adult group, by splitting the dataset in the defined age groups and applying the Mann-Whitney U Test (comparison of 2 subgroups). Results were considered significant if $p < 0.05$. The statistical analysis was performed with SPSS version 25.0 (IBM, Brussels, Belgium).

Results*Influence from age category on fAM conc*

Significant age-related differences could be detected, with adult males (298 (127-829) ng/g DW) showing higher median androgen levels than subadults (153 (108-

336) ng/g DW) and juveniles (156 (119-227) ng/g DW) (Kruskal-Wallis test with Post Hoc Dunn's test; $p < 0.001$) (Figure 1).

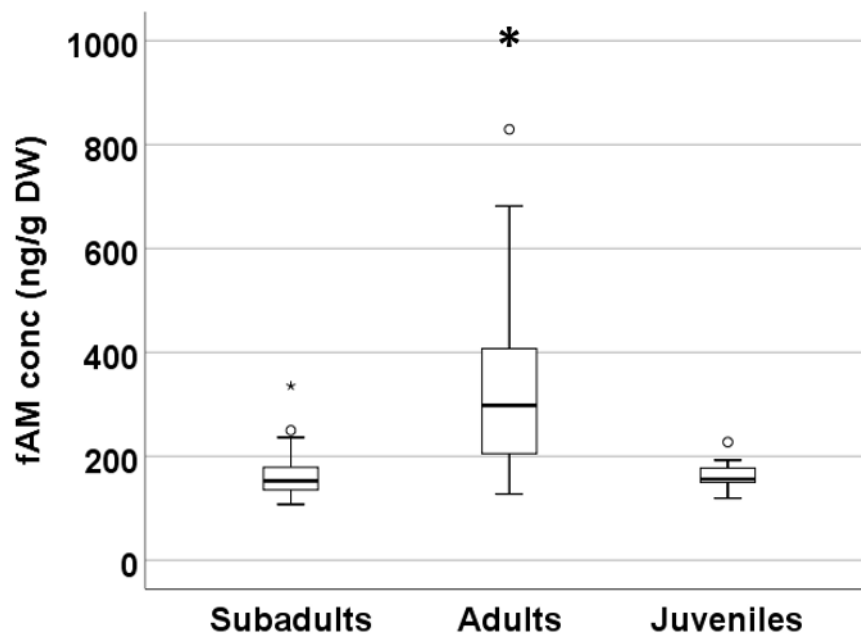


Figure 1. Box plot showing the significant (*: $P < 0.001$; Kruskal-Wallis test with Post Hoc Dunn's test) difference of age related fecal androgen metabolite concentrations (ng/g DW) in fecal samples of juvenile ($n=12$), subadult ($n=28$) and adult ($n=29$) white rhinoceroses bulls (*Ceratotherium simum simum*). Box plot showing the median (continuous horizontal line).

Influence of the breeding behaviour and subsequent offspring on fAM conc

There was a significant difference ($P = 0.04$) between subadults that already demonstrated breeding behaviour, based on mountings (177.56 (152.41-335.55) ng/g DW) compared to subadults without breeding (148.13 (107.69-249.78) ng/g DW) (Mann-Whitney U Test; $p = 0.020$) (Figure 2).

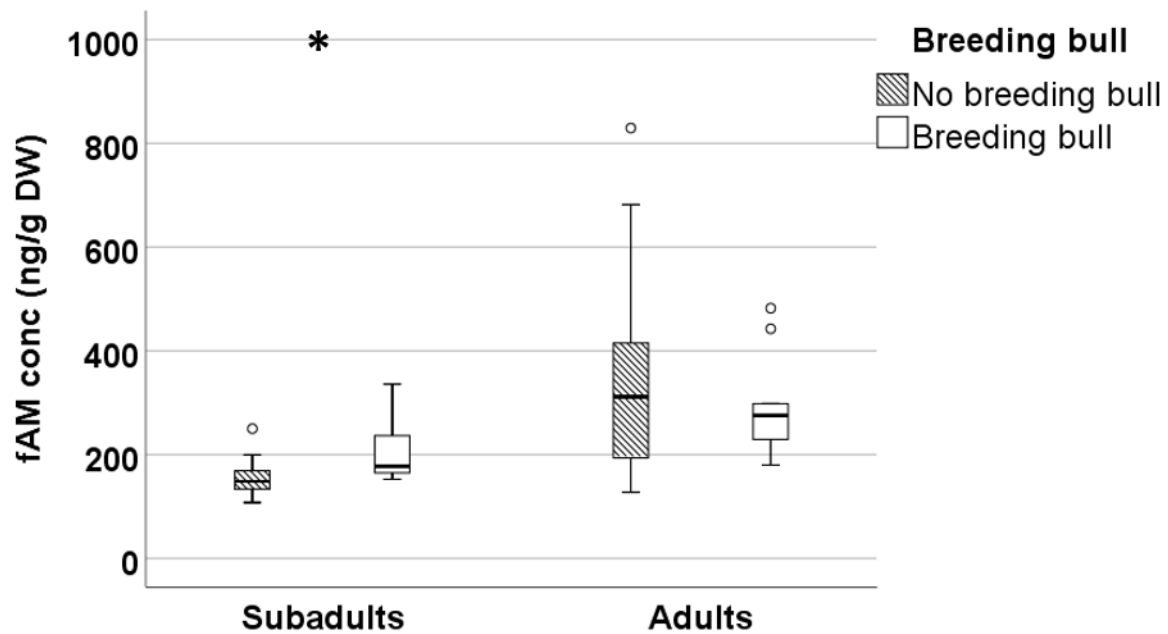


Figure 2. Box plot showing the significant (*: $P = 0.020$; Mann-Whitney U Test) difference in androgen metabolite concentrations (ng/g DW) in faecal samples of subadult white rhinoceroses bulls (*Ceratotherium simum simum*), with ($n=6$) and without ($n=22$) breeding attempts. In adult white rhinoceroses bulls (*Ceratotherium simum simum*) with ($n=9$) and without ($n=20$) breeding attempts there was no significant difference. Box plot showing the median (continuous horizontal line).

In adult males, the difference in fAM between males with or without breeding behaviour was not significant (Figure 2).

Influence from the presence of female adults on fAM conc

No significant differences in fAM concentrations were monitored between males that were kept isolated from females and males that were kept in breeding camps together with adult females (Figure 3).

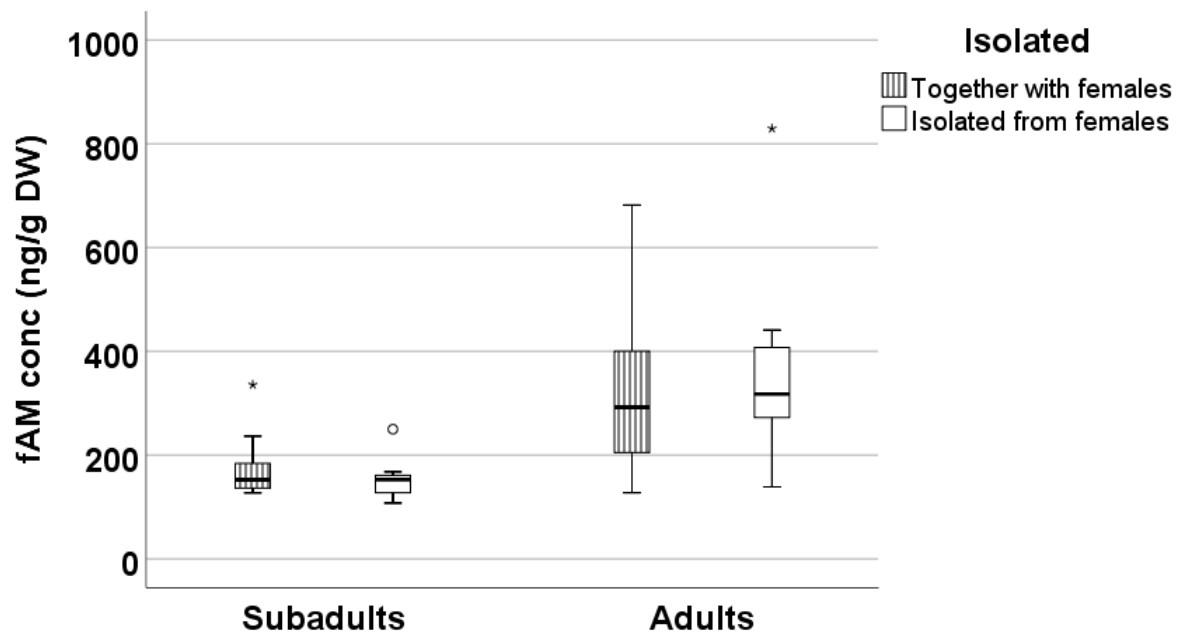


Figure 3. Fecal androgen metabolite concentrations (ng/g DW) of subadult and adult male white rhinoceroses (*Ceratotherium simum simum*) that were kept isolated from adult females (subadults n=7, adults n=10) in bachelor groups (subadult and/or adult males) or kept together with adult females (subadults n=21, adults n=19) in breeding groups (calves, juveniles, subadults and adults). Box plot showing the median (continuous horizontal line).

Discussion

In this study, faecal androgen levels were investigated in a large population ($n = 1,354$) of semi-captive southern white rhinoceros, for the first time in an intensive game-ranch setting. We previously demonstrated that breeding performances in this setting are similar to wild free-roaming and better than most other captive facilities (Ververs et al. 2017). In contrast to the longitudinal study of Kretzschmar et al. (2004) in 5 individual males, this study investigated single samplings of a large number of individuals ($n = 69$) with different epidemiological backgrounds of age, group composition and breeding history and therefore only reflect a trend. The variety within the designated groups might be explained by the single samples.

Already at a subadult age (<71 months), semi-captive males were showing breeding behaviour which is a lot earlier than the age of 10-12 years, as reported for wild free roaming males (Fowler 1978; Shrader & Owen-Smith 2002). Also captive white

rhinoceros males tended to mate earlier than their wild counterparts, from the age of 36 – 72 months, which is probably caused by less competition (Boomsma & van der Sijde ; Lindemann 1982). It is also related to growth-rate and which, in turn, can probably be related to resource availability. In farm animals, the onset of puberty is more closely related to bodyweight than to age. Nutritional levels modulate age at puberty. In this way, overfeeding can result in animals reaching puberty at a younger age (Hafez & Hafez 2013). Optimal nutrition is important, moreover because obese animals do not breed well. In captivity, social structures are often suboptimal because of the lower numbers of animals per facility.

Median fAM-levels in faecal samples of adult males were higher than in the samples of subadults and juveniles. Interestingly, a larger range in fAM levels (127-829 ng/g DW) was found in adults when compared to subadults (108-336 ng/g DW). This might be attributed to incomplete sexual maturation in the subadult group while in the adult group social hierarchy may be responsible for the large range in androgen concentrations between individuals. Another reason could be the fact that only one sample per bull was collected. Sexual stimulation is also known to stimulate GnRH release, LH release and Testosterone production. So sexual or no sexual activity prior to sample collection might be of influence.

Very few reports exist on androgen levels of free roaming and captive male rhinoceroses and none in game-ranched rhinoceroses (Brown et al. 2001; Kretzschmar et al. 2004; Christensen et al. 2009). Individual values of faecal androgen metabolites in this study were remarkably discrepant with another study (Kretzschmar et al. 2004), with the median adult levels in the latter study (57.5 ng/g feces) even lower than the median juvenile levels (156.33 ng/g DW) in this study. This shows that comparing different studies is only representative within exactly the same context e.g. study method and analytical methodology. Furthermore, it indicates that no absolute range of values can be set for age groups in southern white rhinoceros; however, trending differences between age groups remain persistent between studies.

The influence of the presence of an adult female on the testosterone level of the male was studied, but no significant difference could be detected in fAM levels between males kept together or isolated from adult females. This result could be

caused by the low number of animals and the unequal group sizes, or explained by the fact that there was only one sample per bull. For the subadults we observed males kept together with females having higher fAM levels than subadults isolated from females. As presented in an earlier study by Rekwot et al. (2001) on pheromones and biostimulation, this might indicate that these subadults reach sexual maturity at an earlier age because of the presence of females (Rekwot et al. 2001). In the adult bulls roaming together with females we noticed no differences in fAM concentrations when compared to adult bulls isolated from females. In captive black and white rhinoceros there was a positive correlation between testosterone levels and an increasing number of females present. In this same study, average testosterone levels also increased in black rhinoceros males with an increased number of conspecific males. In white rhinoceros however, this specific pattern could not be confirmed (Christensen et al. 2009). Kretzschmar et al. (2004) also showed that elevated levels of androgen metabolites in free-roaming white rhinoceros likely were induced by female presence. In other species, such as the horse, testosterone levels were significantly higher in harem stallions compared to bachelor stallions. Testosterone levels increased when bachelor stallions became harem stallions and decreased quickly when they returned to become a bachelor stallion again (McDonnell & Murray 1995). Territorial wild ungulate males (impala and blesbok) also showed higher testosterone levels than bachelor males (Illius et al. 1983).

In the present study, southern white rhinoceros were kept in large breeding camps within the same area, with a total of more than 1000 southern white rhinoceros present. Camps are fenced off with approximately 10 meter in-between fences, but olfactory, auditory and visual contacts between animals are likely. In camps where there is no visual contact, other southern white rhinoceros always are within close proximity. Therefore it would be interesting to know the range of smell of the southern white rhinoceros and how far olfactory stimulation can play a role in sexual stimulation and androgen levels. Southern white rhinoceros are known to have relatively poor eyesight but very well developed olfactory strength and their communication is primarily mediated by the vocal and olfactory signals (Penny 1987; Cinkova & Policht 2014). Based on sniffing events, the duration of sniffing and the latency of the vigilance posture from the onset of sniffing, southern white rhinoceros were able to discriminate the familiarity and sex of conspecifics. Olfactory cues (and

the vomeronasal organ) could therefore play an important role in social hierarchy (Cinková & Policht 2015). In our study population, dung collected from transport crates was spread in the corners of the camp when new rhinoceroses were introduced to let them settle-in more easily. Further olfactory studies in southern white rhinoceros could therefore be of interest in future research and could play a role in the breeding management of southern white rhinoceros in captivity (Fouraker et al. 1996; Linklater et al. 2006; Linklater et al. 2013). In other species a direct effect of a sex-related odor on neuroendocrine activity (and thus testosterone levels) in a male mammal (hamster) was reported (Macrides et al. 1974).

Contrary to Kretzschmar et al., 2004, the present study was not longitudinal, so seasonal influences could not be studied. Daily samples were taken in the morning, however there could be an influence of daily fluctuations seen in testosterone production. Although faecal samples were collected from the middle of the faecal pile, due to the size of it, hormone levels could be unequally distributed within the pile. Although there are daily fluctuations in testosterone, metabolites assayed in the faeces reflect secretion in the bile over hours and thus peaks are flattened out.

In conclusion, we found that faecal androgen metabolites increase with age and sexual maturity. In game-ranched southern white rhinoceroses (*Ceratotherium simum simum*) there are already subadult bulls that tend to mature early and produce offspring, which is different from white rhinoceroses in free-roaming conditions, but similar to captive settings. A correlation was found between fAM levels of subadult males and subsequent breeding behaviour. No significant difference found between fAM levels of male groups isolated from females or kept with females, suggesting that olfactory and auditory communication between animals from long distance plays a role in the hormonal metabolism of this species.

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CHAPTER 6

Breeding soundness and pregnancy evaluation by ultrasonography in female game-ranched southern white rhinoceroses (*Ceratotherium simum simum*)

Modified from:

Ververs C., van Zijl Langhout M., Otto M., Govaere J., Van Soom A. (2018). Breeding soundness and pregnancy evaluation by ultrasonography in game-ranched southern white rhinoceroses (*Ceratotherium simum simum*). *Theriogenology: under review*.

Abstract

The threatened status of the southern white rhinoceros (*Ceratotherium simum simum*) means that each individual is important for the survival of the species. The reproductive capacity of all remaining animals will be of determinative importance to restoring a population with sufficient genetic diversity. Breeding soundness evaluations in zoological settings have been regularly described, but these evaluations are seldom reported in game-ranched and free roaming rhinoceroses. Breeding problems and reproductive pathologies are common in captive southern white rhinoceroses. Studying this species' reproductive health in different husbandry settings provides crucial information about the underlying causes of infertility. For this study, ultrasound evaluations of the genital tract were performed in immobilised female game-ranched white rhinoceroses (n = 63) kept in large breeding camps (9 ha per adult animal). This study included 17 pregnancies. Pathologies were seen in 11 females, including vaginal discharge (n = 7), endometrial cysts (n = 2) and free fluid inside the uterus (n = 2). Compared to reports of captive southern white rhinoceroses, relatively few reproductive pathologies were identified. Progesterone levels were useful for pregnancy detection starting from 120 days of gestation onward.

Introduction

Non-invasive endocrine studies based on faecal hormone analysis have been widely applied to detect pregnancy in captive southern white rhinoceroses (SWR) and to gain insights into their reproductive status (Roth et al. 2018). Captive rhinoceroses are occasionally trained to stand in a crush so that pregnancy can be diagnosed by ultrasound (Radcliffe et al. 1997; Roth et al. 2004). However, pregnancy monitoring is primarily performed by non-invasive faecal hormone analyses. Progesterone levels rise throughout the pregnancy with a first increase occurring after ovulation during the luteal phase. A doubling of luteal levels appears from around three to five months of gestation (Radcliffe et al. 1997; Schwarzenberger et al. 1998; Patton et al. 1999). Normally, progesterone levels remain high until the end of gestation and suddenly drop in the last days prior to birth or at birth. Continuous endocrine monitoring of the oestrous cycle and pregnancy has resulted in valuable information for planning of natural mating, application of artificial reproductive techniques and the prediction of parturition. However, examination of the genital tract appears to be inevitable for breeding soundness evaluations in rhinoceroses, especially ultrasonographic assessment (Hermes et al. 2006; Hildebrandt et al. 2006).

A large part of the rhinoceros population kept in captivity in zoological institutions suffers from reproductive pathologies. Anovulatory or haemorrhagic follicles, tumours, vaginal discharge and bleeding, uterine cysts, early embryonic death and stillborn calves have been reported in several studies (Hermes et al. 2006; Roth et al. 2018). In most cases, these pathologies could be detected by ultrasound examination of the standing (sedated or trained) animal. Ultrasonography for gynaecological examinations is widely applied in domestic animals (Soede et al. 1998; Medan & El-Aty 2010) and has led to an increased knowledge of the reproductive physiology of many wildlife species (Hildebrandt et al. 2003). With the recent development and application of artificial reproductive techniques such as artificial insemination, embryo transfer and ovum pick-up, often modified from domestic animal procedures, ultrasound examinations have become essential (Hermes et al. 2007).

The ex-situ management of rhinoceroses in game ranches in Southern Africa, where the animals live in semi-wild conditions, differs from Western facilities. For free

roaming and game-ranched southern white rhinoceros in Africa, repeated non-invasive monitoring with faecal hormone analysis is complicated by the natural habitat conditions. In South Africa, for example, rhinoceroses are well cared for and preventively treated or vaccinated if necessary, bought and sold at live auctions, kept in quarantine units before translocations, and immobilised for management purposes like ear notching and DNA sampling. Most privately-owned rhinoceroses undergo regular trimming of their horns to prevent poaching (Emslie & Brooks 1999).

The introduction of breeding soundness assessments in the field, as an incidental or snapshot evaluation during routine management operations, may add significant value by creating a reproductive health certificate. These assessments might also help to determine the types of abnormalities and pathologies that occur in this species in captivity. In terms of breeding management, sales and animal welfare, reproductive health certificates could increase an animal's value, decrease the risks of translocating pregnant females and avoid unnecessary translocations of infertile animals.

This study investigated the use of ultrasound to perform breeding soundness evaluations and early pregnancy detection in extensively managed semi-wild rhinoceros populations.

Materials and methods

Ethics statement

The authors confirm that all methods were conducted in accordance with the relevant guidelines and regulations and that all necessary permits were obtained from the South African authorities before dehorning was performed. All procedures were carried out as part of routine health management and good veterinary practices. Retrospective data analysis was performed with permission from the owner, and none of the animals were handled for the sole purpose of this research project.

Study area and animals

The female southern white rhinoceros in this study ($n = 63$) were part of a confined, private, game-ranched breeding herd ($n = 1,354$) and were kept in large breeding camps (9 ha per adult animal). The group consisted of nine juveniles, nine subadults,

five young adults and forty adults. The study was conducted between February and December 2014 with follow-up that continued until the last animal from the study calved. The game ranch was located in a semi-arid climate. The facility consisted of several adjoining properties divided into breeding camps with 25 to 70 animals per camp, depending on the size and location. The rhinoceroses were given additional feed when natural grass was limited. Supplementary feed consisted of alfalfa (lucerne) (8 kg) and pellets (5 kg) daily during the dry winter season (June to August). The amounts were then decreased until adequate rainfall allowed the animals to return to a natural grazing diet. Natural mating occurred without human intervention other than controlling the breeding camp numbers and monitoring the carrying capacity of the field and camp with the minimal surface per rhinoceros set at 9 ha per animal.

When an animal was identified from a distance by its ear notches, anaesthetics were administered with a remote dart system. Immobilisations were performed throughout the year but only in cool and dry weather. Adult animals were darted in the musculature of the neck, shoulder or quadriceps with 4 mg of etorphine hydrochloride (Captivon, 9.8 mg/mL, Wildlife Pharmaceuticals, White River, South Africa) and 40 mg of azaperone (Stresnil, 40 mg/mL, Janssen Pharmaceutica, Johannesburg, South Africa); subadults received 2 to 3 mg of etorphine and 20 to 30 mg of azaperone; and calves >1 year received 0.5 to 2 mg of etorphine and 5 to 20 mg of azaperone. Once the procedures were completed, 20 mg of naltrexone (Trexonil, 50 mg/mL, Wildlife Pharmaceuticals) per 1 mg of etorphine was injected intravenously to antagonize the opioid component of the anaesthesia. During the rest of the day, the treated rhinoceroses were kept within eyesight and supervised by rangers using binoculars.

Mounting behaviour

Mounting behaviour was recorded when observed. One groom was assigned per camp to carry out observations throughout the day. The grooms reported to management three times a day. Females in estrus showed obvious signs of mounting, such as scuff marks on their flanks and back from the legs of the bull. The identity of the bull and the cow was recorded to monitor the fertility of each bull and to predict the possible parturition dates for the cow. Each calving was recorded. The age of the rhinoceroses was classified according to the following categories: juveniles

(21 to 30 months); subadults (31 to 54 months (females) and 31 to 71 months (males)); young adult females (55 to 71 months); and adults (72 months and older). Each breeding camp consisted of 1 to 3 adult breeding bulls with 25 to 30 adult females, 10 to 25 calves and 10 to 25 subadults.

Placement and preparation for examination

Once a rhinoceros was immobilised, any external reproductive abnormalities were investigated. Vulva conformation and colour, vaginal discharge, and udder health were scored. The animals were put in sternal recumbency to maintain good respiration. Rectal faecal balls were removed manually with a lubricated rectal glove. A portable ultrasound (Ibex® Pro) connected to a goggle-style headset (InSite® 2 Video Headset) with a linear transducer (L6.2 MHz) was used. When necessary, mostly in large adult females, the Ibex® Customizable Extension (I.C.E.®) was used to reach the ovaries. A methodical protocol was conducted, comparable to the one used in the rectal examination of large domesticated animals such as cattle and horses. The cervix was palpated, followed by inspection of the uterine corpus and the uterine horns. Uterine oedema was scored, according to the equine scoring system, from zero to four, with zero being none and four being maximum grade of oedema. Finally, the ovary was reached (Ververs et al., 2015).

Faecal sample collection, storage, processing and extraction

For females determined to be pregnant by ultrasound, faecal sample collection and processing was used to determine progesterone levels. Actual calving dates were confirmed at the end of the study.

Faecal samples were deep frozen (-20°C) within 2 hours after collection. The deep frozen samples were lyophilized, pulverised and sifted using a mesh strainer to remove fibrous material. Approximately 0.05 g of the faecal powder was then extracted with 80% ethanol in water (3 ml) by vortexing for 15 minutes and subsequently centrifuging for 10 minutes at 1500 g. The resulting supernatants were transferred into micro-centrifuge tubes and stored at -20°C until analysis. The cross-reactivities of the antibody used in the study are described by Szdzydy et al. (2006) (Szdzydy et al. 2006).

Faecal progesterone metabolite assays

The extracts were measured for immunoreactive faecal progesterone metabolites (fPM) using an enzyme immunoassay (EIA) for 5 α -pregnan-3 β -ol-20-one, which has been shown to provide reliable information on the reproductive steroid hormone pattern by reflecting the total progestogens in different mammalian species (Szdzyu et al. 2006; Ahlers et al. 2012). The EIA used a polyclonal antibody against 5 α -pregnan-3 β -ol-20-one-3-hemisuccinate-BSA and a 5 α -pregnan-3 β -ol-20-one-3-hemisuccinate-peroxidase label. The coefficients for the intra-assay variance ranged from 6.37% to 6.65%, and the coefficients for the inter-assay variance ranged from 10.87% to 12.82%. The EIA sensitivity was 9 ng/g dry weight (P9-IZW EIA). To adjust for water content variations, the fPM concentrations were expressed as mass/dry mass of faecal extract.

Results

Clinical observations as part of the breeding soundness evaluation

Seven of the examined females showed vaginal discharge (Figure 1), two of them while pregnant (Figure 1, F2 and F5). Discharge was viscous and yellow, except in one non-pregnant female that showed a bloody vaginal discharge (which had been ongoing for seven months).

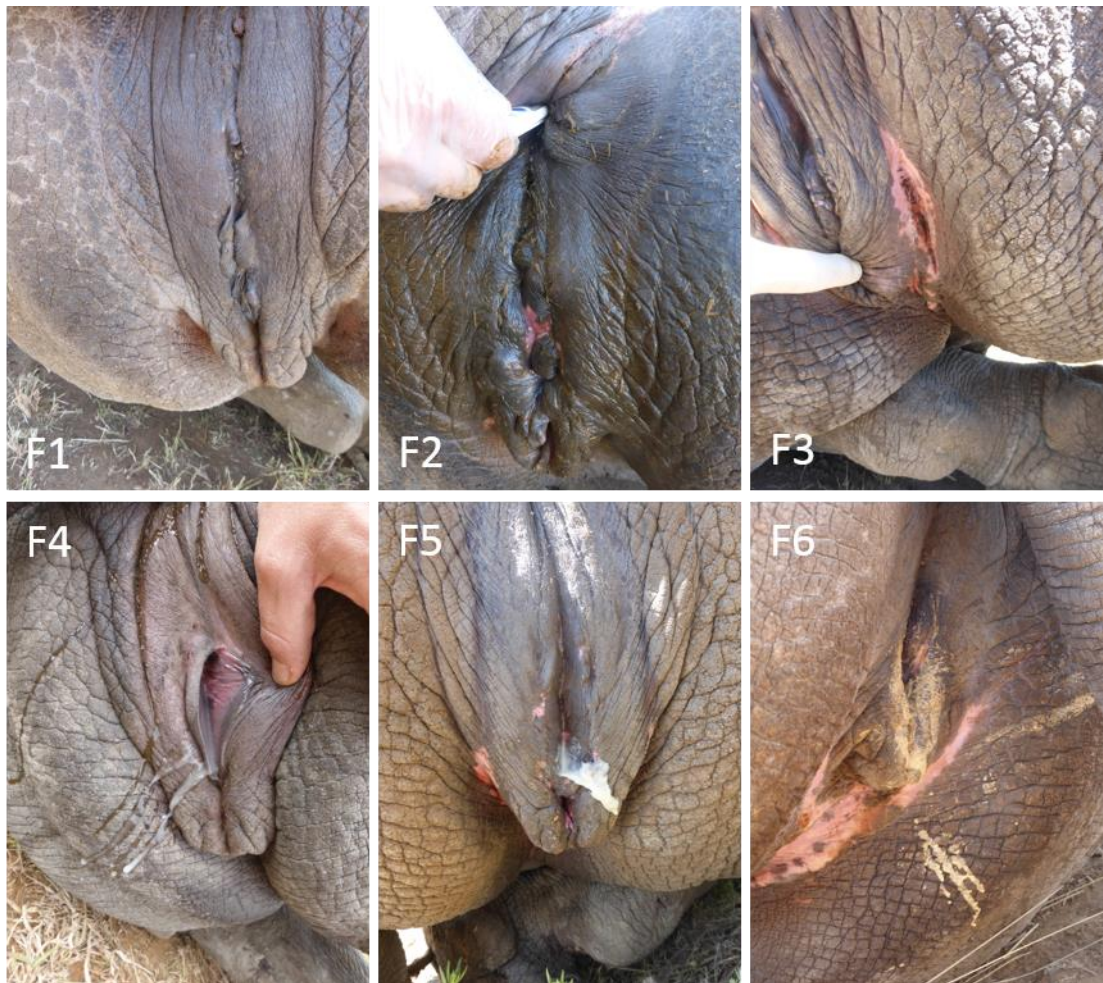


Figure 1. Features of the vulva of six southern white rhinoceros females (*Ceratotherium simum simum*). Normal vulva (F1); bubble-like vulva (F2); normal vulva with tick-bite lesions next to the vulva lips (F3); vulva with clear mucous discharge (F4); vulva with yellow purulent discharge (F5); dried yellow crystals, presumably after urination or as a result of previous yellow purulent discharge (F6).

When the photographs in Figure 1 (F1-6) were taken, the rhinoceros in F2 was 12 months pregnant (and gave birth to a healthy calf); the rhinoceros in F4 was in oestrus; the rhinoceros in F5 was carrying an embryonic vesicle and produced a healthy calf 16 months later; and the rhinoceros in F6 was in heat, and conception led to a calf born 16 months later.

Pathologies of the reproductive tract could be detected in four females (4/63). Two females, with parities of zero and four, showed uterine endometrial (intraluminal) cysts, and two females, with parities of zero and two, showed free fluid in the uterus (Table 1).

Table 1. Female southern white rhinoceroses (*Ceratotherium simum simum*) with reproductive pathologies detected on ultrasound. E3 and E4 refer to the grade of the oedema in a grading system of one to four, where E1 is the absence of uterine oedema, and E4 is maximal uterine oedema.

Female	Pathology	Comments	Age at examination	Parity
1	Endometrial cysts	2 small cysts, 5 mm diameter	25 months	0
2	Endometrial cysts	Multiple cysts, uterine corpus and horn, 10-25 mm diameter	144 months (estimated)	4
3	Free uterine fluid, > 1cm	Uterine oedema (E3)	36 months	0
4	Free uterine fluid, > 1 cm	Uterine oedema (E4)	132 months (estimated)	2

Pregnancy detection by ultrasound

Out of 40 adult females, 15 adult females (Table 2; C and G to T) were detected as pregnant by visualization of a foetus, and 3 adult females (Table 2; D, E, F) had free uterine fluid (localized, anechogenic, > 5 cm), but no foetus could be clearly visualized. Out of the 18 pregnant and presumed pregnant females, 6 were pluriparous (being pregnant with their third calf), 9 had previously had one calf, and 3 were nulliparous. In the remaining 22 females, a normal uterus was visualized. Of these animals, 18 were confirmed to be not pregnant, and 4 were classified as presumed not pregnant, but the examination could not be completed due to time limitations. Since only mating and calving dates were recorded, a wide variation in the gestation period is likely. Because detection of the ovulation and mating times was based on circumstantial evidence (scuff marks) and most females mated more than once, it was only possible to estimate the duration of the pregnancy.

Table 2. The faecal progesterone metabolites (fPM) ($\mu\text{g/g}$ dry weight) of 5 non-pregnant and 15 pregnant female southern white rhinoceroses (*Ceratotherium simum simum*) with their ultrasound diagnoses and their gestation periods based on an average duration of 16 months (488 days). EED: early embryonic death.

Females	Gestational age (days) (based on 488d pregnancy)	Days to subsequent calving	Faecal progesterone metabolite (fPM) ($\mu\text{g/g}$ DW)	As determined by ultrasound
A	-	-	2	Cyclic
B	-	-	2.8	Cyclic
C	-	EED	2.4	Pregnant (vesicle)
D	-561	1049	1.9	Undetermined
E	-221	709	6.2	Undetermined
F	-200	688	2.3	Undetermined
G	-31	519	3.1	Pregnant
H	26	462	2.7	Pregnant (vesicle)
I	36	452	4.5	Pregnant
J	44	444	3.7	Pregnant
K	73	Aborted	3.8	Pregnant
L	87	401	6.3	Pregnant
M	100	388	137.40	Pregnant
N	146	342	43.6	Pregnant
O	201	287	65.5	Pregnant
P	358	130	144.2	Pregnant
Q	368	120	289	Pregnant
R	372	116	135.7	Pregnant
S	375	113	172.9	Pregnant
T	466	22	126.4	Pregnant

Two females showed a clear embryonic vesicle of 19 mm and 21 mm, respectively (Figure 2). Uterine contractions with embryo movement were visible on the ultrasound, indicating migration of the embryo in the early stages before attachment. Seven of the females were pregnant within the first 120 days of gestation (Table 2 and Figure 2). The most advanced gestation was at 466 days of pregnancy, based on the recorded mating time. Uneventful calving of this dam occurred 22 days later (Table 2).

Female C (Table 2 and no 1 in Figure 2) never gave birth to a calf. On the ultrasound, the uterus appeared thick and possibly oedematous. This might be an indication that the pregnancy was already failing. She mated again 72 days after the examination; this also did not result in a birth. Female K (Table 2) aborted 107 days after the examination and the foetus was found.

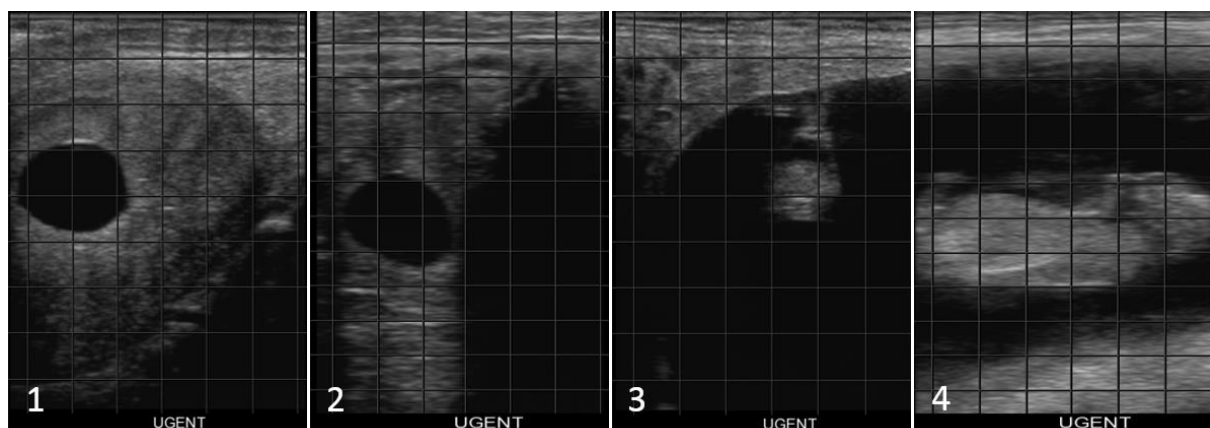


Figure 2. Ultrasound images of 4 different pregnancies. 1 and 2: Embryonic vesicles (spherical) of two early pregnancies in a southern white rhinoceros (*Ceratotherium simum simum*), 19 mm (no 1) and 21 mm (no 2) respectively. Contraction of the uterus was visible, and the vesicle was migrating. 3: Dorsal orientation of the foetus can be seen (approximately day 36 of pregnancy), and a heartbeat was detected. The foetus measured 14 mm (no 3). 4: A southern white rhinoceros foetus at approximately three months of gestation (no 4). One square on the ultrasound image represents 1 cm².

Faecal progesterone metabolite concentrations

Faecal samples were analysed (Table 2). Fifteen females (C and G to T) were determined to be pregnant by rectal ultrasound examination. The fPM levels increased throughout pregnancy but only exceeded the cyclic luteal phase levels in 8 out of 15 pregnant females, all of which had progressed beyond 87 to 100 days of

gestation. Pregnancy was detected in females C and G to L by ultrasound, but this could not be confirmed by faecal analysis. Females E and L both had fPM levels of between 6 to 7 µg/g DW, but only female L appeared to be pregnant.

Early pregnancy in female K was detected by ultrasound (uterine fluid and embryo), but she aborted 107 days after the ultrasound examination at a gestational period of 180 days. No further tests were conducted. Female K mated 75 and 106 days after she aborted, and she conceived again. She gave birth to a healthy calf at 605 days after the abortion (after a gestation period of 499 to 530 days).

Females A and B served as a control group. Both were found to have a small uterus by transrectal ultrasound, indicating that they were barren. This was confirmed by fPM levels. No calf was born to either female within three years of the examination.

Discussion

In contrast to the findings of captive animals described in the literature, we identified few reproductive pathologies in this study's game-ranched southern white rhinoceros. Zoological institutions often hold rhinoceroses in small numbers that limit reproduction frequency; these holdings are also typically part of an ageing population that is suffering from reproductive pathologies (Hermes et al. 2004). The incidence of reproductive disorders is greater in nulliparous females and is positively correlated with age. These disorders are regarded as age-related consequences of long non-reproductive periods (Hermes & Hildebrandt 2011). Consequently, the reproductive health of these animals is probably not representative of the species. In captivity, the first reproductive lesions are often detected at the age of 16 years. Therefore, pregnancy in young animals is often regarded as a prophylactic measure against reproductive disorders (Hermes et al. 2006; Hermes & Hildebrandt 2011). The South African semi-captive southern white rhinoceros in this study were able to exhibit more natural reproductive behaviour since they were held in large groups and roamed an extensive area.

All ultrasound examinations were performed on the rhinoceroses in sternal or lateral recumbency. Basic reproductive findings such as the stage of the ovarian cycle, the presence of endometrial cysts and cystic endometrial hyperplasia, pregnancy, uterine fluid, uterine tumours and other pathologies could be detected, when present, during

an examination of 25 minutes. Oestrous cycle and pregnancy monitoring in captive southern white rhinoceros has been conducted by several institutions where animals were trained to enter a chute for an ultrasound procedure (Radcliffe et al. 1997; Hildebrandt et al. 2006; Roth et al. 2018). Breeding soundness evaluations have detected reproductive tract pathologies (such as endometrial cysts and tumours of the uterus, ovaries and testicles) in captive white rhinoceroses with long non-reproductive periods as well as in Indian Rhinoceros (Hermes et al. 2004; Hermes et al. 2005; Hermes et al. 2014; Hermes et al. 2016). In game-ranched and free roaming rhinoceroses, few reproductive pathologies have been reported.

In our study population, with 60% of the females being older than six years, the ultrasound examinations did not reveal any internal pathologies except for uterine endometrial cysts (2/63) and limited amounts of free uterine fluid (2/44 non-pregnant females). Based on the number and sizes of the cysts, it could not be determined if they were a possible cause of reproductive failure. In horses, large cystic areas in the endometrium have been shown to interfere with uterine blood flow and to decrease fertility (Ferreira et al. 2008). Free uterine fluid could be a sign of endometritis that impairs fertility and requires treatment (LeBlanc & Causey 2009). Depending on the age and parity of the female as well as the location and size of the pathology, some uterine conditions can be linked to the aging process and lengthy exposure to an oestrogen-dominant physiological environment. Due to pregnancy failures and cycle irregularity, captive female rhinoceroses will experience three to ten times as many oestrous cycles as wild females, and they will spend less time pregnant or in lactation anoestrus (Hermes et al. 2006; Penfold et al. 2014). Two of the females with reproductive pathologies in this study were 11 and 12 years old, while the other two females with reproductive pathologies were 2.5 and 3 years old (Table 1). In these cases, an endometrial swab could refine the diagnosis (Riddle et al. 2007), although passing the tight folded cervix of the female rhinoceros is a challenging procedure. It is difficult to judge the presence of a genital pathology based only on vaginal discharge (Figure 1).

A simple grading system to assess vaginal conformation has been developed to create consistency between observers (Carter 2007). According to this system, the bubble-like swellings are graded from zero to three with zero representing no swelling (anoestrus) and three representing very swollen bubbles (oestrus). Ovulation is

confirmed based on elevated progesterone concentrations (Carter 2007). When we applied this grading system in our study, we could not always relate the grade of the vulva to the cycle stage. Moreover, vaginal discharge, if from a pathological origin, did not seem to influence fertility (Fig 1).

In this study, we detected two embryonic vesicles (19 and 22 mm) with concurrent uterine contractions and vesicle migration that could be visualized on ultrasonography. Early pregnancy detection by ultrasound is commonly performed in many domestic species, but not in wildlife. It has rarely been studied in captive rhinoceroses (Radcliffe et al. 1997; Roth et al. 2004). The embryonic vesicles in captive rhinoceroses have been detected as early as 15 days post ovulation, and migration of the vesicle through the uterus has been observed in subsequent examinations. The embryo has been visible from day 23 onwards, and heartbeat has been detected at day 26 (Radcliffe et al. 1997). Similar findings were observed in the Sumatran rhinoceros, where an embryonic vesicle of 3 to 5 mm was seen on ultrasound as early as 14 days after mating. Mobility of the embryonic vesicle was noted, and it expanded to 10 mm by day 16. A heartbeat was detected by day 26 (Roth et al. 2004). In the domestic horse, the embryonic vesicle and its migration through the uterus can be detected as early as nine days post ovulation, and migration continues until day 17 when fixation occurs (Leith & Ginther 1984).

Early embryonic loss has been seen in rhinoceroses held in captivity. Often the cause is attributed to endometritis (Radcliffe et al. 1997; Roth et al. 2001). In this study, one of the pregnant females (Table 2; female C) who was confirmed pregnant by the presence of an embryonic vesicle (Figure 2, no 1) never gave birth to a calf. She mated several times afterward, indicating that she was cycling. One of the early pregnant females aborted 2.5 months after examination (Table 2; female K). That cow immediately conceived again and gave birth to a healthy calf, indicating that endometritis was a less likely cause of the abortion.

The levels of fPM in this study indicated that there were non-pregnant cycling females (Table 2; females A and B), probably in the luteal phase. Without conception, levels of fPM rise 7 to 9 days after ovulation of the dominant follicle and remain elevated for 19 to 22 days in southern white rhinoceros. After conception, the progesterone levels remain elevated beyond the cyclic luteal rise (Radcliffe et al.

1997)[3]. Our results of cycling females showed similar trends to those found in previous studies (Schwarzenberger et al. 1997; van der Goot et al. 2015). Brown et al. (2001) found average baseline faecal progesterone concentrations of 1.22 ± 0.41 $\mu\text{g/g}$ and peak luteal phase levels ranging from 3 to 24 $\mu\text{g/g}$ (Brown et al. 2001). Comparing absolute values between studies is difficult due to differences in sample manipulation and analysis, but fPM trends might provide valuable information.

Three females (D, E and F) were detected as potentially pregnant by ultrasound examination due to localized anechogenic fluid, although no conceptus was detected and the examinations could not be completed due to time constraints (table 2). Their fPM levels appeared to be below pregnancy levels at that stage of gestation, and there were no confirmed births. Therefore, these females were not pregnant at the time of the ultrasound examination but successfully mated at a later stage (and calved at 1049, 709 and 688 days after the ultrasound examination). The uterine fluid was likely misinterpreted. This demonstrates that a pregnancy can only be confirmed or excluded after a complete and thorough examination. Sometimes animals need to be repositioned during recumbent examination, particularly when they are positioned in sternal recumbency. Only detection of the embryo with a heartbeat or a live fetus confirms a viable pregnancy, but, of course, abortion can still occur at a later time. Due to the large size of the rhinoceros' reproductive tract, a linear probe is not preferable. A convex probe, with 30 cm of penetration, is more suitable.

Females G to T were confirmed pregnant by ultrasound examination and, except for female C who underwent early embryonic death and female K who aborted 107 days after the ultrasound examination, a healthy calf was born within the physiological gestation period (± 488 days). All 15 pregnant females were at different stages of gestation (Table 2). This table shows that progesterone levels in pregnant southern white rhinoceros start to rise above the luteal phase levels at around 90 to 120 days of gestation, similar to previous studies (Brown et al. 2001; van der Goot 2015). Therefore, pregnancy can be detected at an earlier stage by ultrasound examination than by fPM, and ultrasound may also provide additional information about the genital tract and the cycle stage. In this study, 47% (7/15) of the pregnant females were determined to be pregnant by ultrasound at a gestation stage where it was impossible to discriminate between pregnancy and the luteal phase by fPM levels.

Longitudinal follow-up with fPM analysis is recommended. However, in game-ranched and free-roaming rhinoceros, this is very difficult.

Conclusions

Concurrent hormone analysis confirmed ultrasonographically-identified pregnancies. Without ultrasound, it is more difficult to determine the exact timing of ovulation, to differentiate between a non-pregnant luteal phase and an early pregnancy, and to identify early embryonic death. Breeding soundness evaluations of game-ranched southern white rhinoceros under field conditions are useful, practical and can be implemented during routine management procedures, such as translocation, dehorning, ear-notching and DNA sampling. These evaluations will contribute to the general reproductive information of southern white rhinoceros. Finally, these evaluations will benefit future attempts at artificial reproductive techniques and the conservation of all rhinoceros species.

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CHAPTER 7

GENERAL DISCUSSION

The main objective of this thesis was to describe the reproductive performance of southern white rhinoceroses, particularly in game-ranched settings. We defined certain aspects of the reproductive physiology under game-ranched conditions, together with the different factors influencing it, and this has led to a list of key reference values for reproduction in game ranched southern white rhinoceros. The mere fact that we got access to the world's largest rhinoceros facility was an prerequisite to perform these investigations, which have shed a light on possible causes of poor reproductive performance in captivity until now and on the importance of social structures, for example on male fAMC. We have demonstrated that breeding soundness evaluation during regular management interventions can produce valuable insights in the reproductive status and could help to detect reproductive pathologies, that are more prone to develop in captivity.

Africa is home to a variety of wildlife, including some large mammals also known as megafauna. Megafauna like the southern white rhinoceros, which typically are animals with high longevity, slow population growth, and low mortality rates, since there are very few or no natural predators capable of killing an adult. However, their large size and extended habitat makes them vulnerable to human overpopulation and once declining in numbers, they are more sensitive to become threatened because of their slow population recovery rates. Looking at the southern white rhinoceros, huge conservation efforts have already been made in the past. It is important to situate the history of conservation of the rhinoceros, in order to understand the importance of game-ranching, and the value it may have for species conservation.

Southern Africa's conservation of its wildlife and their intensified wildlife breeding is different to conservation elsewhere in Africa. In the year 1800 about 1 million rhinoceros lived in Africa and Asia. In 1900, the southern white rhinoceros had been hunted almost to extinction, with about 50 individuals remaining in a single South African reserve. The population slowly recovered in the middle of the 20th century. As a result, relocating breeding groups of white rhinos became possible, both to other national parks in South Africa and to private game reserves. In 1968, the first legal trophy hunt on a white rhino was authorized. In 1960, the first 3 southern white rhinoceros were translocated from Imfolozi to Kruger National Park. Kruger national park greatly contributed to the recovery of the species and housed more than 10,000 individuals in 2014.

However, by the 1980's, a strange disjunction emerged. In 1982, the Natal Parks Board (South Africa) had a list price for a living white rhino of about 1,000 South African Rand. The average price paid by a hunter for a rhino trophy that year was 6,000 South African Rand. Soon after, the auctioning of rhinoceroses started. In 1991, property rights and markets entered the story in a different way. Before 1991, all wildlife in South Africa was treated by laws as *res nullius* or un-owned property. To reap the benefits of ownership from a wild animal, it had to be killed, captured or domesticated. This created an incentive to harvest, not to protect, valuable wild species. This also meant that even if a game rancher paid for a rhino, the rancher could not claim compensation if the rhino left his property or was killed by a poacher. Therefore a new legislation was included: the theft of Game Act of 1991. This policy allowed for private ownership of any wild animal that could be identified according to certain criteria such as a brand or ear tag. The combined effect of market pricing through auctions and the creation of stronger property rights over rhinoceroses changed the incentives of private ranchers. It all of a sudden made sense to breed rhinos rather than shoot them as soon as they were owned. To visualize the differences made to conservation by these issues of property rights and incentives we compare the African black and white rhinoceros. There is a strong contrast between white and black rhinoceros population numbers between 1960 and 2007. Black rhino mostly lived in African countries with a weak or absent commercial wildlife market such as Kenya, Tanzania and Zambia. In 1960 about 100,000 black rhinos roamed across Africa, but by the early 1990s poachers had reduced their numbers to less than 2,500. Therefore, unprotected wild rhino populations are rare to non-existent in modern Africa. Another reason for the decline is habitat loss and changes of feeding regimes. The only surviving African rhinos remain either in countries with strong commercial wildlife markets (South Africa and Namibia) or in intensively protected zones. Unfortunately a strong demand for rhino horn remains, and especially since about 2008, rhinos across Africa face a risk of illegal poaching (Muir-Leresche & Nelson 2000; Sas-Rolfes 2012).

Illegal wildlife trade is a global conservation issue that targets, besides the few high-profile species like rhinoceros, tigers and elephants, thousands of other species (e.g. timber, medicinal plants, fungi, shark fins, pangolins and many more). There

are growing scientific and policy debates about the most effective conservation intervention. Increased enforcement demands reduction strategies, provision of alternative livelihoods as well as approaches that are more market-based and incentive-based (Biggs et al. 2013; Duffy 2014; Phelps et al. 2016). Notwithstanding, different sources of a single product may be legal, while others are illegal with its consequences for legal prosecution makes it very difficult, time-consuming and inefficient to control. For instance, farmed versus wild-collected specimens, organisms collected within or outside of official quotas and individuals traded domestically versus internationally (Phelps et al. 2016). Improving relations with local communities to help solve the human-wildlife conflict as well as increasing incentives for conservation creates the necessary backbone for successful enforcement. Wildlife is better conserved where net benefits (financial and non-financial) to individuals of local communities of retaining it are greater than net benefits (financial and non-financial) of engaging in illegal wildlife trade. Intervention should combine both 'top-down' enforcement and diverse community-engagement approaches (Cooney et al. 2017). The contribution of hunting and legal trade of wildlife products remains a difficult issue. International politics and wildlife conservation organizations are in constant debate about the pros and cons of certain regulations. Due to conservation management, which is basically interfering with or manipulating nature, truly wild populations remain scarce. In the case of the rhinoceroses, the question around legalization of the horn trade does not seem to have a unequivocal answer. Long term conservation strategies are of course the best option (in an ideal world) and should be kept in mind, but this is maybe not feasible. Survival of the species should be the main goal, and if temporary well-regulated trade can play a role, it should be taken into account as well. Education of the end-user of wildlife products and solutions for human-wildlife conflicts in rural areas should however be the key strategy to solve the poaching problem. Other strategies can only serve to gain time in the battle against species extinction. Preservation of wildlife areas is the most important means of protecting wildlife and endangered species. Here private game reserves, where animals are largely free-ranging, play a huge role.

Due to a lack of funding for public parks as well as limited scope to enlarge the state-protected areas, some conservationists are turning to private landowners for

solutions. Although there are certain benefits to it, there are also limitations and challenges related to private wildlife ranching as a tool for conservation. Some of the risks might be that tourist preferences might drive the industry and that predators are persecuted to protect valuable wildlife. Clear guidance, guidelines and support for wildlife ranchers are requested to realize conservational benefits and minimize economic losses of ranchers (Cousins et al. 2008, 2010).

Breeding of white rhinoceroses: better in zoological institutions or in wildlife-ranches ?

In zoological institutions all over the world captive breeding of southern white rhinoceroses has been a challenge for years (Versteeg 2017). Population figures show only a slow increase in recent years. The growth rate of the captive population is around 1%. If we look at European captive rhinoceroses (EAZA registered), there are currently 310 individuals (Figure 1). Only since 2013 there is an absence of import from animals outside Europe.

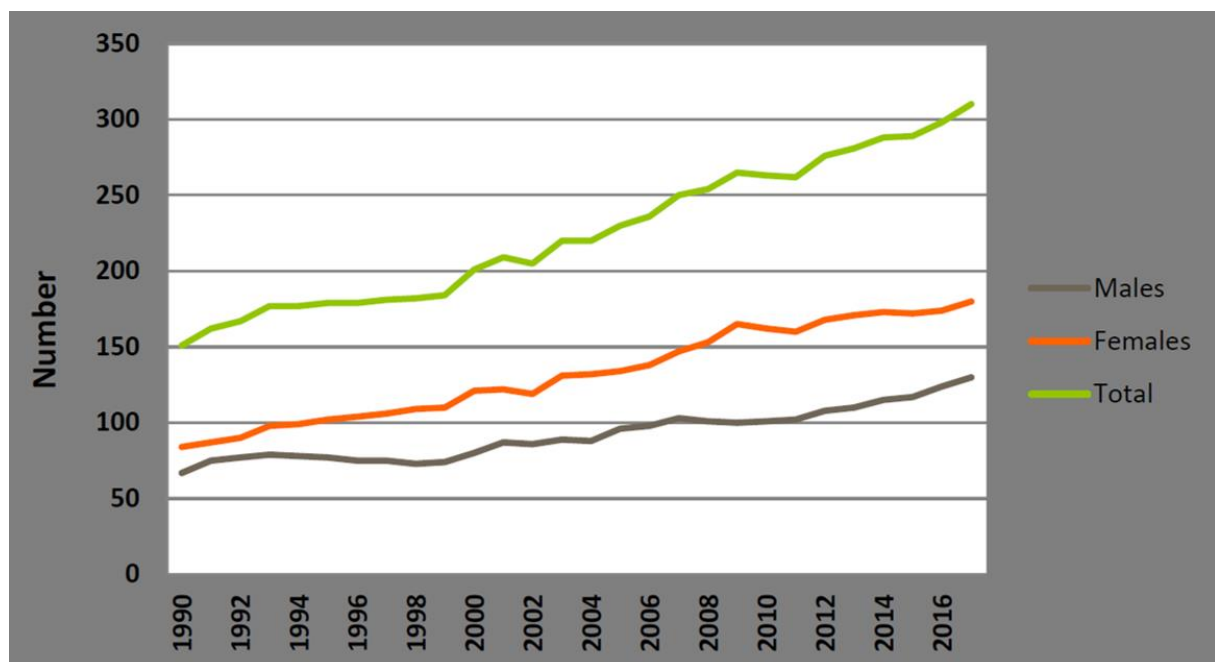


Figure 1. Captive population numbers of EAZA registered white rhinoceroses.

One of the important details regarding population growth, shown in figure 2, is the high percentage (21%) of newborn calves that die before reaching the age of 1 year (this holds true for both sexes). To maintain the population growth of 1%, a minimum

of at least 9 births is necessary (based on the current population size), taking into account the probability that a pair will breed is set to 50%. Only the last three to five years this has been achieved. The peak reproductive age is between 7 and 27 years of age (Figure 2)

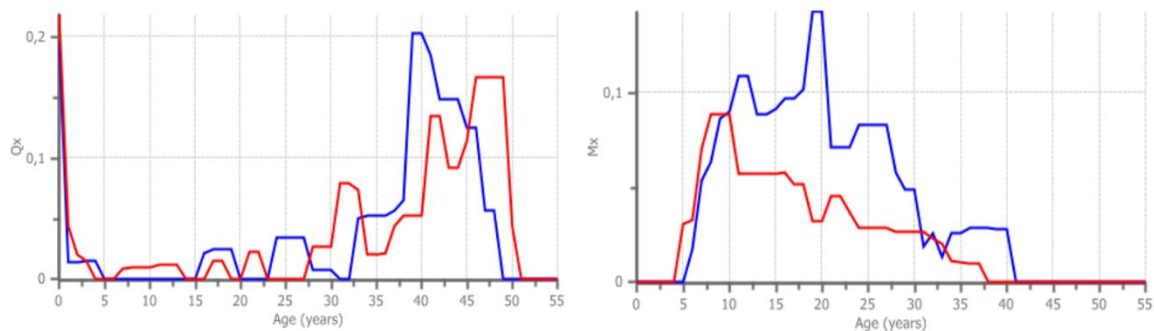


Figure 2. The left graphic shows the age specific mortality and the right graphic shows the age specific fecundity. Red lines show females and blue lines show males (Versteege 2017).

The percentage of animals breeding in captivity has increased in recent years, but still reproductive pathologies cause high percentages of non-breeding females (table 1).

Table 1. Breeding and non-breeding captive southern white rhinoceros in EAZA institutions (Versteege 2017)

Breeding status (%)	2009	2011	2015	2017
Breeding	21	23	36	43
Non-breeding < 10 yrs	38	38	29	24
Non-breeding 10-20 yrs	11	16	20	20
Non-breeding >20 yrs	30	24	15	13

In this dissertation we aimed to give an overview of current features of reproduction and assisted reproduction in southern white rhinoceros and used the horse, the closest domestic relative, as a model (**chapter 1**). Research on large numbers of wild animals are limited when compared to domestic species. For the first time we determined reproductive parameters for southern white rhinoceros based on a large number of game-ranched individuals.

Reproduction in southern white rhinoceros has been studied by a broad audience of scientists, but mainly in relatively small groups of captive animals or widely spread groups of free roaming animals in their natural habitat. The reproductive cycle of captive rhinoceros has been studied and revealed a lot of irregularities in length and reproductive success (Roth 2006; Roth et al. 2017). In free roaming white rhinoceros there is still limited knowledge, but a recent study showed physiological, regular fertile cycles (van der Goot et al. 2015). Therefore it seemed interesting to study game-ranched rhinoceroses, which are in between free roaming and captivity. Our study site, the world's biggest rhinoceros ranch, created an opportunity to observe and work with the largest semi-captive population of white rhinoceros in the world, that once might become of great genetic importance. The farm contains more than 5% of the total population left worldwide and is larger than total number of southern white rhinoceros living in captivity to this date (Foose & Wiese 2006).

(Slow) Population growth rates

The first study conducted within this doctoral thesis determined the reproductive parameters of a large number of southern white rhinoceros within an identical geographic location (**chapter 3**). The parameters observed are herd growth, calving rate, artificial growth, age at first calving, inter-calving interval and the effect of translocation on subsequent calvings. Furthermore we looked at seasonal influences and the effect of rainfall on the number of births and sex ratio of offspring.

Herd growth – Calving rate – Artificial growth – Age at first calving

Reproductive behaviour and performance, as well as expression of appropriate social behaviour is one of the most common obstacles to conservation breeding. Previous research stated that decreased reproductive performance in captivity might be associated with the limited possibilities to exhibit normal reproductive behaviour

patterns (Swaisgood et al. 2006). With limited herd sizes, managed in rather small areas that are often totally different from the natural conditions, the annual growth rate is negative (-3.5%) while growth rates in wild populations are still 6-10% (Emslie & Brooks 1999). According to the biodiversity management plan for the white rhinoceros in South Africa 2015 – 2020, the current number in National, Provincial and private game reserves is 18,900 southern white rhinos. This counts for 93% of the total population worldwide. Around 4536 of these rhinoceros accounts privately owned in South Africa, which is 24% of the South African population (Knight et al. 2015). Therefore, an appropriate housing and management system appears to be crucial, as well as a correct diet, to achieve success in captive breeding systems. Polygamous breeding, where dominant males have access to several adult females, seemed to be required (Owen-Smith 1975; Lindemann 1982; Fouraker et al. 1996). Since our study facility is registered by the South African authorities as a captive breeding operation, it shares the same regulations with zoological institutions. However, due to the size of this facility and the number of individuals, there is polygamous breeding indeed and both adult males and females have several mating options.

Over 8 years, a total of 562 calvings were recorded in our study population. The number of eligible females calving every year was expressed as an annual calving rate. Since the gestation period of the southern white rhinoceros is 16 months on average, we introduced the biennial calving rate as well. This number is a similar measure of performance as the inter-calving interval is, however the difference is that the average percentage of adult females calving annually and biennially also includes the eligible females that have not calved. Whether one should look at annual or biennial calving rate in animals with a gestation period of more than 12 months is open for discussion. Since both the total herd growth and population growth in our facility was not only influenced by calves being born at the facility but also by newly acquired animals that were introduced (moved inside from other facilities), it is difficult to compare these growth rates with those of previous studies with wild or captive populations. To avoid misinterpretation of the parameter population growth, we introduced the new parameter herd growth in this dissertation, since it deals with game-ranched rhinoceros. Furthermore, since more than 30% of the southern white rhinoceros are owned and managed by the private sector, and the South African

sector owns more southern white rhinoceros than there are in the rest of Africa, animals are bought and sold from one owner to another. Herd growth of game-ranchered southern white rhinoceros is therefore the closest related parameter to compare with population growth in previous studies that looked at wild white rhinoceros. A population growth of 10.5% was previously documented as a theoretical maximum for the species (Owen-Smith 1992). In our study the herd growth was on average 7%, which was mainly caused by the slow start of the breeding operation where population growth was mainly caused by new arrivals. The parameter 'age at first calving' was determined since it provides more information about health of a growing population. With a median age at first calving of 7 years (83.21 months), the breeding herd showed results in accordance with rapidly growing populations where females may have their first calves as young as at 6.5 years of age (Owen-Smith & Smith 1973). Other studies showed ages at first calving of 5.6-8 years (Patton et al. 1999) and 7.4 – 10.1 years (Rachlow & Berger 1998; Skinner et al. 2006). During the course of this study, the age at first calving did not increase significantly when numbers and density of animals increased.

Inter-calving interval

We registered a median inter-calving interval of 29.25 month, where SADC guidelines classify less than 30 months as good to excellent (Du Toit 2006). Moreover, our result seems to be an overestimation, since some animals imported with an incomplete calving history, exhibited a longer inter-calving interval. Animals with higher parity still showed regular inter-calving intervals of 25-27 months, despite regularly undergoing anaesthetic procedures for management purposes. In previous studies free roaming rhinoceros showed an average inter-calving interval of 30 months (Owen-Smith & Smith 1973), or even of 34.8 (low density) to 39.6 months (Rachlow & Berger 1998), whereas white rhinoceros in captivity showed a larger variation in inter-calving period from 19.5-29.5 months (91 births from 30 females) (Lindemann 1982) or 34 months, calculated on 33 calving intervals of animals with various parity (Skinner et al. 2006).

Effect of translocation on subsequent calving

In our study a large percentage of the females calved within 16 months after arrival at the facility. The calving rate continued at the same pace before and after

translocation. Therefore, we concluded that either the method of translocation was not overly stressful or stress did not affect the established pregnancies. Abortions were extremely rare in translocated females

Seasonal influences per month on the number of births

The results of 562 calvings showed a clear seasonal calving pattern of the game-ranched southern white rhinoceros, with a peak of 28 calves being born in one month (March 2015). A significant increase in number of calvings was seen during December – April in comparison to the rest of the year.

Effect of rainfall on the number of births and sex ratios

Historic rainfall (mm/day) data from the area did not show a significant effect on the calf sex ratio, when based on the moment of conception. In our study, no significant skewed progeny sex ratio was detected. A skewed sex ratio, as described for the first time in the hypothesis of Trivers and Willard, could reduce the future rhinoceros population reproductive success with males outnumbering cows (Trivers & Willard 1973).

(Slow) Horn growth and the effects on fertility parameters

In the **fourth chapter** of this dissertation we investigated and described horn growth of the rhinoceros. The horn is currently the most valuable part of a southern white rhinoceros and unfortunately a widely trafficked wildlife product. As a result, a rhinoceros is worth more dead than alive. Due to the massive increase of poaching in recent years, with over 1000 individuals killed over five consecutive years, different efforts have been made to solve this problem. Unfortunately, there is no single solution, but one of the precautions taken in managed situations is the preventative trimming of the horn (Figure 3). Because there are different types of area and management systems to keep white rhinoceros, not one strategy guarantees a 100% success rate. Therefore each setting requires a unique approach to prevent poaching. Security options of the property play an important role and influence the outcome of prevention measures. There are no long term studies available yet to prove that this technique is effective, and short term reports about dehorning as a conservation strategy are sometimes contradicted. However, in combination with a reduction in end-user demand, education and extensive anti-poaching security it

might take some poaching pressure away in certain reserves and game-ranches. In this way it helps to save time to develop long term conservation strategies. If applied in a correct way, the horn will grow back just like human fingernails (Figure 4).

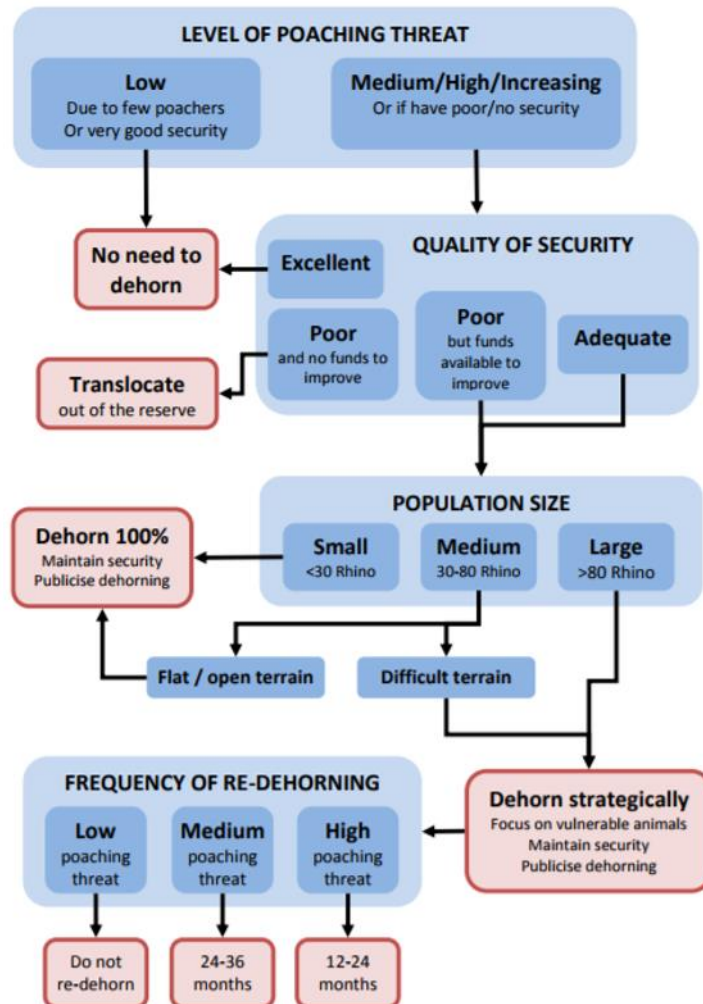


Figure 3. Shows possible dehorning strategies in relation to the level of poaching threat (Lindsey & Taylor 2011).

Horns, both anterior and posterior, can be removed without severe pain (although further studies would be good to look into details) during a safe chemical immobilisation procedure, when executed according good veterinary practices by avoiding the penetration of the growth plate and not cutting too close to the germinal layer (Figure 4). Other precautions can be horn infusion with poison (not without risk for the animal), translocation of animals to secure locations and of course high-end security measures.

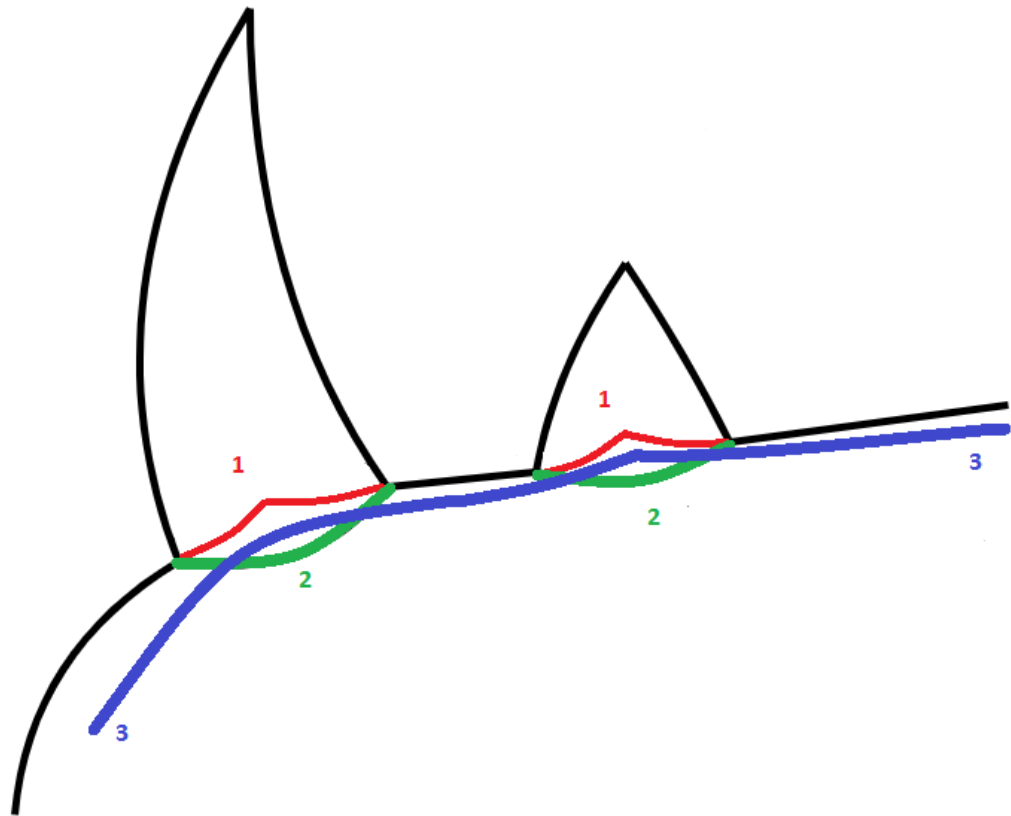


Figure 4. This anatomical draw shows the growth plate (Red, number 1), the skin-horn junction (Green, number 2) and the skull (Blue, number 3).

Until the start of this study, very little research on the consequences of dehorning on reproductive performance of southern white rhinoceros had been conducted. Up-to-date growth parameters for anterior and posterior horn growth and regrowth could substantially support management decisions in regard to anti-poaching precautions. In this retrospective study of 2044 dehorning events performed on 671 unique identity females and 747 unique identity males, all studied under equal conditions and the same geographic location, we detected clear influences of sex on the horn length, weight and circumference. In this study, no negative effects could be detected for reproductive performances of these dehorned rhinoceroses, when compared to other rhinoceroses that had not been dehorned. We also established growth and re-growth indices and compared them with the growth of bovine claws and equine hoofs as well as with growth studies in free roaming white and black rhinoceroses (**chapter 4**).

In this study all individuals were dehorned on a regular basis. It would however been interesting to look to what extent there was still no negative influence from dehorning when only a certain percentage of a population would have been dehorned. In some of the bigger reserves with free roaming rhinoceros it is not feasible to dehorn every single animal, due to remote locations and high costs involved. In such cases it could be feasible to dehorn only the individuals on the perimeter of the reserve/park that suffer the highest poaching threat. In these situations there would be an overlap between rhinoceroses with an intact horn and individuals that were dehorned. It would be interesting to investigate whether these animals still maintain their previously established hierarchies and mating structures, when compared to fully dehorned populations or fully horn-intact populations. Dehorning rhinoceroses as a routine management intervention, especially with the aim of harvesting horn for commercial benefits, is still part of an intense public debate. Without making a statement in this discussion about horn trade, we might decide, based on the reproductive performances of the studied population, that regular dehorning does not harm reproductive outcomes. Further research into stress related parameters and long term outcome would however be interesting. Dehorning under game-ranched conditions in a more or less controlled environment might also be compared with dehorning events in free roaming settings. Based on this study, we cannot conclude that sexual selection and male choice of mating is related to the presence of an intact horn. Previous studies on male choice suggest that males may maximize their reproductive success by defending areas containing more grassland habitat. The amount of grassland in a males' territory predicted that females would use it (White 2006). Owen-Smith (1973) proposed that females may select a male for mating simply by entering his territory during her fertile period. Overlap of home ranges in (semi) captive settings could influence mating strategies. And although white rhinoceros are a sexually dimorphic species in which males exhibit different mating strategies, territorial males achieve greater reproductive success than non-territorial males. Fighting among males is believed to be the main cause of mortality in free roaming settings (Rachlow et al. 1998). The presence of an intact horn might play a role in this territory establishment.

Although nutrition was not taken into account in our study and is beyond the scope of this thesis, we suggest that nutrition likely has an influence on the growth of the horn

and that it is worthwhile to include this factor in future studies. Tiunov and Kirilova (2010) studied the horn of the ancient woolly rhino and found that each transverse band consisted of a pair of layers or zones. One of which is lighter and denser while the other is darker and looser. The isotopic composition of progressively laid down keratinous tissues such as hair, horn and hooves can provide valuable information on their diet and environment. They found that the distance between layers became shorter when age increased. They also investigated the isotopic composition of carbon and nitrogen of the keratinous tissue and related it to the expressed transverse layers of the horn. The nutritional influences might hold especially in captive settings with either constant additional feeding or seasonal additional feeding in times of insufficient natural food sources available. For instance, studies on phytoestrogens suggested negative influences on reproductive performance. Horn growth however was not taken into account in these studies (Tubbs et al. 2012; Tubbs et al. 2016; Tubbs et al. 2017).

Faecal androgen metabolite concentrations in males of different age and social hierarchy

In the **fifth chapter** we aimed to study the relationship between fAMC of individual males and their breeding behaviour. The study animals were part of a confined private game-ranched southern white rhinoceros breeding herd and were kept in large breeding camps (9ha per adult animal). In each individual camp there were 25-70 animals housed, depending on the size and location. To avoid overpopulation and inbreeding in camps due to successful breeding, sub-adults (2.5-6 yr) were removed from their natal camps and placed in new camps to establish new breeding herds or bachelor groups of surplus males. Each breeding herd consisted of 1-3 dominant adult breeding bulls, 25-30 adult females, 10-25 sub-adults (males and females) and 10-25 juveniles and calves (males and females). In accordance with Kretzschmar et al. 2004, performed on free roaming southern white rhinoceros, we looked at male androgen levels and related them to breeding behaviour and success in our game-ranched set-up. Although the situation is fully managed, natural behaviour within the different camps is stimulated as much as possible. The management aimed to minimize the number of translocations for every animal to a maximum of 1 in a lifetime. We collected faecal samples rectally during events when male rhinoceroses were immobilised for management purposes, or we collected fresh faecal samples

from males that we saw defecating during observation. Individual males were identified by the use of ear tags (observation) and microchips and ear tags (immobilised).

Significant age-related differences in faecal androgen metabolite concentrations could be detected, with adult males showing higher median androgen levels than subadults and juveniles. Subadults that showed already breeding behaviour had significantly higher androgen levels than subadults that had not bred yet. The presence of adult females did not show a significant influence on the faecal androgen metabolite concentrations of the males, however, complete isolation of males could not always be guaranteed due to olfactory influences from females in nearby camps. Due to the limited number of individuals and the fact that only one sample per individual was taken, future research should contain more samples to create a longitudinal study that could study the seasonal influences on fAMC as well.

Ultrasound appearance of the genital tract

In the **sixth chapter** of this dissertation we examined the genital tract during an immobilisation and detected pregnancies by ultrasonography. For this purpose we used a portable ultrasound machine (Ibex Pro®) with extender for large females (Ibex I.C.E. ®) and a linear transducer (L6.2) (Figure 5).



Figure 5. Breeding soundness evaluation with a portable ultrasound machine.

Ultrasound evaluation of the genital tract as part of a breeding soundness evaluation was performed in game-ranched males ($n=57$) and females ($n=63$) that were anesthetized for routine management purposes. Of the females, 17 were detected pregnant, 7 showed vaginal discharge, 2 of them had endometrial cysts and 2 showed some free fluid inside the uterus.

As for pregnancy detection, faecal hormone analysis is often used as a non-invasive method. However, in game-ranched settings, animals are frequently translocated or anesthetized for management purposes. During these procedures we used ultrasonography to detect pregnancies between 0-4 months and compared ultrasound findings with faecal hormone analysis. Here we showed that these pregnancies often only have luteal-level progesterone values and that 'early' pregnancies cannot be reliably diagnosed during these stages of gestation. Ultrasonography therefore is indicated to be used more often during breeding soundness evaluation when this can be implemented in management procedures like

DNA sampling, ear notching or horn trimming events. Pregnant females are of high value in conservation management, especially during the ongoing poaching crisis as for auction sales to establish new breeding groups.

Female genital tract pathologies could hardly be detected in our reproductive sound study population (Ververs et al. 2017). In captive populations, many adult females show reproductive pathologies, often related to non-breeding or non-pregnant status (Hermes et al. 2004). In this study we could only detect minor pathologies like uterine free fluid and uterine endometrial cysts. Additional vaginal discharge was detected more often, but could not be related to reproductive tract pathologies and was also seen in healthy pregnant females that delivered a healthy calf. The aetiology of vaginal discharge could be of interest for future studies.

Hypothesis confirmed

Our hypothesis was that we could learn from semi-captive game-ranched white rhinoceros to help saving them from extinction. Since the population studied is the largest captive population worldwide, we considered it a perfect environment to determine statistical relevant reproductive parameters. We believe that our results can benefit both breeding *ex situ* in captive situations as well as rhinoceros conservation breeding *in situ* in free roaming situations.

Future perspectives

Genetic variety will become a key issue to maintain a sustainable rhinoceros population in the future. Trying to stop further poaching (by education efforts and anti-poaching actions) might be the biggest challenge, but optimizing the breeding and increasing knowledge on the reproduction of these magnificent creatures might be a solution to compensate current losses and to maintain a strong gene pool.

Since pregnancy diagnosis by hormone analysis in faeces is not always accurate within the first five months of pregnancy, ultrasound is the method of choice to determine early pregnancy. Future challenges could be to find other (earlier) endocrine markers of pregnancy in either urine or faeces. Pregnant females are of higher conservation priority and higher economic value. However, to perform and tolerate an ultrasound examination, the animal needs to be sedated, anesthetized or

trained. Until now, it is not studied if frequent anaesthesia or sedation has an influence on (early) pregnancy in the white rhinoceros.

Despite the rhinoceros being considered a non-seasonal breeder by some scientists (Owen-Smith, 1973), there are indications that season might influence rhinoceros reproduction (Patton et al., 1999) (chapter 3). It may be important to consider these factors when thinking of future breeding in zoological institutions. Due to the small number of animals used in most studies, it is sometimes difficult to find the true causes for reproductive failure.

Several aspects of rhinoceros reproduction have been studied (Roth 2006; Roth et al. 2017). However, most research and previously discussed techniques have been reported in captive rhinoceros in zoological settings. Breeding rhinoceros in a more natural habitat, under natural circumstances might lead to different results. We therefore encourage future studies in game-ranched animals, either longitudinal as well as ‘snap-shot’ events. A lot of reproductive problems as described above are mainly seen in captive born male and female rhinoceros. Groups kept in most zoological institutions are small and stable when compared to animals living in the wild. The choice for breeding is often limited to certain animals within the zoological institution. Males and females are frequently kept together in the same group or within visual or olfactory distances, with translocations and new introductions being less frequent. Studies have shown that sub-adult mature bulls are often suppressed by an older dominant male in the group, which may affect semen quality. Evaluation of different group settings will be necessary in further research, as improvement of reproductive performance after management changes has already been proven (Hermes et al. 2005). In wild rhinoceros, strong ‘friendships’ have been described between different animals (Smith & Norman 1975). A better reproduction rate would be expected when individual animals have the freedom of choice. Adult females in the wild tend to keep their calf on foot until the next calf is born. In zoological institutions, it is often more difficult for sub-adults to voluntarily move into a separate (bachelor) group, as they are mostly kept within the same enclosure. Could this be the reason for the early occurrence of sexual puberty in captivity, due to a lack of competition? Could this be the reason why certain sub- or infertile males and females become fertile (again) when transferred to another institution or back to their natural habitat? All these questions, as well as the breeding successes so far, prove that

breeding rhinoceros in captivity is challenging. Recent endeavours of EAZA and the white rhino EEP show already some improvement. To support breeding results, each females between the ages of 6 to 20 years, which fails to reproduce, is checked for reproductive pathologies. Furthermore, young females are transferred out of their maternal herd. This will stimulate natural breeding. In free roaming settings adolescent females also disperse to nearby regions, eventually joining other adolescent animals or adult females without calves. This resulted finally in a positive natural growth of the captive population, albeit only limited. Creating a better understanding of rhinoceros reproductive health would be of great help, especially, when more and more reproductive techniques will be applied. Nowadays in particular, when ongoing poaching threat continues at its current rate, knowledge from this species that is not (yet) close to extinction will offer future opportunities for the white as well as for nearly extinct (whether or not closely related) species (e.g. black (*Diceros bicornis*), Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*).

Furthermore we already gained good insights in reproductive performance of a large breeding facility as described in this dissertation. However, thanks to the increased intensified breeding operations in southern Africa there is much more to learn for zoological institutions outside Africa. The facility studied in this dissertation exceeded captive numbers of white rhinoceros worldwide. Nutrition, both in captive and semi-captive is considered highly important for future research.

Since dehorning and trimming of the horn of rhinoceroses to decrease the ongoing poaching threat was implemented in the early 90's (Kock & Atkinson 1993), many private game reserves as well as some of the national and provincial parks nowadays implement preventive dehorning together with security measures as an anti-poaching strategy. Even a couple of captive rhinoceroses were poached for their horn and some zoological institutions in the world decided to trim the horn of their captive rhinoceroses to prevent it from being poached. Adult animals are considered of great genetic value and even with decreased fertility results, are still more important in a safe environment as breeding animals at risk. Our aim was to evaluate the risk of dehorning and its influences on fertility. Furthermore we wanted to look into horn growth rates to establish an 'ideal' or 'optimal' dehorning strategy as long as this is necessary to prevent poaching. In this study the conventional technique of dehorning

was performed, also because horn stumps were collected and stockpiled for future purposes (in case of legalizing the trade). Purely as an anti-poaching measure, another dehorning technique can be applied as well. This is the so called K(ock)M(orkel) method, where first the major stump is removed with the chainsaw and afterwards the stumps are carefully ground with an angle grinder to remove any remaining horn (Figure 6).



Figure 6. Two different dehorning techniques. The conventional method (left) versus the K(ock)M(orkel) method (right).

In future studies it would be interesting to compare anti-poaching results and conservation effect of both these techniques when applied on a group of rhinoceroses that live under the same conditions. Since horn re-grows again, we suppose that the main difference between the techniques is that, as less horn is left, dehorning with the KM method perhaps will keep poachers away somewhat longer. Additionally, the perspective from the poacher could also be studied more in detail. Financial incentives for poaching are still increasing due to rising prices. Growing relative poverty between areas of supply and centers of demand as well as aggressive enforcements of trade controls (in particular bans), can increase profits and lead to the involvement of organized crime. Therefore it is urgent that we should incentivize and build capacity within local communities to conserve wildlife (Challender & Macmillan 2014). Table 2 shows a final overview of approaches that might lead to solutions of this multifactorial problem.

Table 2. Possible approaches to stop poaching of rhinoceros for their horn

Approach	Outcome	Difficulties
To educate the end-user of rhinoceros horn through social marketing programs	Will solve the problem for once and for all	Will probably take centuries to become effective
Incentivize and build capacity within local communities	Conserving species will be more profitable than poaching them	Sufficient wages need to be paid and poachers need to be educated- It may be better to focus on people who may become poachers then to try and convert poachers
Establish environmental courts to improve the successful prosecution of couriers and buyers	Less recidivists	Law system has to be stable and reliable
Ranching and wildlife farming; keep game ranched rhinoceros and dehorn them at regular intervals for regular supply on a legalized market	Increase of rhinoceros numbers and incentives for private ownership	Will it be ethically correct to 'farm' rhinoceros for their horn?
Prevent trafficking of wildlife product and improve border controls	Less illegal trafficking	Challenging process that requests worldwide cooperation of crimescene institutions
Synthetic horn production	Flood the market and fulfil the demand	End-user might not be interested in a synthetic horn

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SUMMARY

Despite an increase in number of births, the worldwide growth of the population of the white rhinoceros is slowing down mainly due to anthropogenic causes, such as poaching and habitat loss. Assisted reproduction is one of the methods of preserving the valuable genomes of these animals from being lost and assists in breeding them in captivity to maintain the specie(s) numbers and provide an option for possible reintroduction into the wild. Wild rhinoceroses are difficult to examine and most of the current information available on their reproductive characteristics is gained from captive rhinoceros populations. Nevertheless, nowadays knowledge about rhinoceros reproduction is still insufficient.

The rhinoceros belongs to the odd-toed ungulates (Perissodactyls) group, like the horse and the tapir. Therefore we proposed the horse as a suitable model to study reproduction and artificial reproductive techniques. Studies on the general reproduction in domestic large animals has been widely performed and physiological parameters are established long time ago. However, in many wild species, this is often not the case. Limited studies are available and not many research groups are involved in this domain. With the rise in semi-captive game ranched institutions, wild animals can be studied more easily while they are still kept in their close-to-natural habitat.

The purpose of this PhD thesis was to improve general knowledge on reproductive parameters of the African southern white rhinoceros and find reference values for game-ranching white rhinoceroses. We studied different aspects of the world's largest breeding facility of game-ranching white rhinoceroses that could contribute to the general knowledge of captive as well as wild breeding programs for this endangered species.

In **chapter 1**, the general features of reproduction and assisted reproduction are reviewed. The chapter explains classification and population numbers currently living in captive, semi-captive and wild settings. Since the domestic horse is the closest domestic relative to the rhinoceros and assisted reproductive techniques are often adapted from current knowledge in domestic species, the reproductive anatomy and breeding performances in equines are compared to these of rhinos. The oestrous

cycle of the white rhinoceros in captivity is reported to have a lot of irregularities which are often only studied in limited study groups. To the authors opinion, some aspects of captive breeding would therefore greatly benefit from studies in large game-ranched conditions.

Chapter 2 describes the aims of this dissertation. The general aim was to improve conservation breeding for a species that is threatened, the African southern white rhinoceros. However, other species of the Rhinocerotidae like the African black rhinoceros (*Diceros bicornis*), the Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) which live in Asia, could also benefit from increased knowledge. They all differ in population numbers and level of endangerment, with some being listed as critically endangered (black, Javan, Sumatran) to vulnerable (Indian) and some being listed as near threatened (white). We also studied the influence of regular horn trimming (as a measure of poaching prevention) on the breeding performance and had a closer look at early pregnancy diagnosis during breeding soundness examinations.

Since there is a variety of reproductive cycles described in captivity and reproductive performances in captivity are rather poor, we studied in **chapter 3** breeding characteristics of a large group of semi-captive game-ranched white rhinoceros. In this study, performed between 2008-2016, we analysed data from 1.354 rhinoceroses kept in a 8000 hectares game-ranch. Since there is a dynamic growth of this population, not only caused by new-born animals but also by animals bought into the property, we introduced parameters like the annual herd growth, the annual calving rate and the biennial calving rate. In total 562 calving events were included in this dataset. The females had a median age of 83.2 months at their first calving and the multiparous females had inter-calving intervals of 29.2 months. These parameters were not influenced by translocation of the animals. Multivariate models showed a clear seasonal calving pattern with a peak in births during December-April. We could not find a significant skewed sex ratio in our study, which is in contrast with previous studies. By weather observations we could not find rain influences or seasonal influences on the sex of the calves.

Nowadays safety precautions of poaching prevention exists of patrolling by anti-poaching units as well as preventative trimming of the rhinoceros horn. Horn is made

of keratin like human fingernail and equine hoof, so if trimmed in a correct way, it will grow back. **Chapter 4** gives insights in the influences of regular dehorning ($n=2044$) on a rhino population in a South African game-ranch. We determined horn growth reference values and studied the possible influences like age, number of dehornings and timing of dehorning on the growth of the anterior and posterior horn (Figure 1). Males in this study showed a higher growth rate of horn (2.36 ± 0.082 g/day) compared to females (1.74 ± 0.073 g/day). On average, the dehorning interval was 587 days. Since there are also some zoos that decided to trim the horns of their rhinoceros because of safety precautions, this study also contributes to that captive management part.



Figure 1. Measurement of the front horn length (left) before trimming the horn (right) at a safe distance from the base (photo: Brent Stirton, National Geographic).

In game-ranch settings, rhinoceros are kept into breeding camps, allowing them to establish social hierarchy and a family structure to exhibit natural courtship and breeding patterns. Surplus males are kept in bachelor camps. In contrast, in captivity, where social structures and eligible partners are imposed, males tend to breed at a younger age when compared to their wild counterparts. Therefore we looked in **chapter 5** at differences between age-groups and whether breeding could be related to testosterone levels. We found a significant difference between the different age groups, with adults (≥ 72 months) showing higher testosterone levels than subadults (31-71 months) and juveniles (21-30 months). Testosterone levels in the breeding versus non-breeding groups only significantly differed in subadults but not in adults. Because of poor sight, olfactory perception could play a key-role and is worth looking into for future studies.

In the game-ranch facilities where wildlife breeding is often done more intensively, we looked at the possibilities to implement routine breeding soundness examinations in males and females. Individuals are more often anesthetized for management purposes like translocation, DNA sampling, horn trimming or ear notching. **Chapter 6** shows the use of ultrasonography during breeding soundness evaluation to detect reproductive tract pathologies in males (n=57) and females (n=63). In this study we hardly found any pathologies which is different to the captive population worldwide that includes many non-reproducing females with severe reproductive tract pathologies. We also compared results of ultrasonography in 17 pregnant females to faecal progesterone metabolites. We showed that early pregnancies (up to 4 months) cannot be accurately detected by the use of faecal hormone analyses. Progesterone levels in these early pregnancies equal levels of progesterone during luteal phase of (non-pregnant) cyclic females. Ultrasound examination therefore can be of additional value and can routinely be implemented in management interventions and translocations. It would also generate useful information on the reproductive tract of white rhinoceros that eventually benefit new approaches for artificial reproductive techniques in captive, semi-captive and wild populations.

In the last chapter (**chapter 7**) of this thesis, the results of the different studies are discussed. We concluded that research on game-ranched rhinos can definitely contribute to the southern white rhinoceros population currently left worldwide. There are some similarities between captive and wild environments, but also some discrepancies with captivity regarding reproductive performances. Since regular trimming of the horn is nowadays implemented in bigger national and private reserves as well, lessons can be learned from game-ranched facilities like the one studied in this dissertation. Since we are losing southern white rhinoceroses at an alarming rate, future studies into reproductive techniques are recommended. However, before implementing assisted techniques in this wonderful creature, basic reproductive physiology still needs to be further unravelled.

SAMENVATTING

Ondanks de toename in aantal geboortes neemt de wereldwijde groei van de witte neushoornpopulatie af door antropogene factoren, zoals de stroperij en het verlies van leefgebied. Gecontroleerde en geassisteerde voortplanting is een van de methoden om waardevolle genetica te behouden en ondersteunt de voortplanting in gevangenschap met eventuele herintroductie in het wild. Gezien de neushoorn in het wild moeilijk benaderbaar is, werd tot op heden de meeste informatie rondom de voortplantingseigenschappen opgedaan bij in gevangenschap levende neushoorns. Tot op heden is de wetenschappelijke kennis over de voortplanting van de neushoorn nog altijd summier en niet eenduidig.

De neushoorn behoort tot de onevenhoevigen (Perissodactyla), net als het paard en de tapir. Het paard kan daarom als geschikt model fungeren bij studies naar de (geassisteerde) voortplanting. Gedomesticeerde dieren zijn uitgebreid bestudeerd geweest in het verleden, waardoor fysiologische parameters bekend zijn. Dit is bij wilde dieren vaak niet het geval. Er zijn relatief weinig data beschikbaar en slechts enkele onderzoeksgroepen bestuderen deze. Door de toename van 'in situ' beheerde en gecontroleerde populaties, kunnen (semi) wilde dieren tegenwoordig beter bestudeerd worden in een leefomgeving die dicht bij hun oorspronkelijke natuur ligt.

Dit proefschrift heeft als doel om de algemene kennis rondom voortplantingsparameters bij de Afrikaanse zuidelijke witte neushoorn te verbeteren en het bepalen van referentiewaarden voor intensief gehouden witte neushoorns. Hiervoor werden er verschillende aspecten bestudeerd in 's werelds grootste, intensief gehouden groep van witte neushoorns. Deze informatie kan bijdragen aan reeds bestaande fokprogramma's voor deze bedreigde diersoort *in situ* en *ex situ*.

In **hoofdstuk 1** wordt een overzicht gegeven van de huidige literatuur omtrent de (geassisteerde) voortplanting bij de neushoorn. De classificatie alsook de populatie aantallen van in gevangenschap, semi-gevangenschap en wild levende dieren worden weergegeven. Daar het paard als model fungeert, wordt hiermee de vergelijking gemaakt. Veelal worden technieken geëxtrapoleerd vanuit de

gedomesticeerde diersoorten, omdat in dat domein reeds meer onderzoek verricht is. De oestrische cyclus van de witte neushoorn in gevangenschap is hoofdzakelijk bestudeerd in kleine groepen en blijkt erg onregelmatig. Bepaalde aspecten van de kweekprogramma's *ex situ* zullen daarom veel baat kunnen hebben bij studies in grotere populaties *in situ*.

Hoofdstuk 2 beschrijft de voornaamste doelen van dit proefschrift; het verbeteren van de fokprogramma's voor een diersoort dat met uitsterven bedreigd is, namelijk de Afrikaanse zuidelijke witte neushoorn. Maar ook de andere ondersoorten zoals de Afrikaanse zwarte neushoorn (*Dicoreas bicornis*), de Indische (*Rhinoceros unicornis*), Javaanse (*Rhinoceros sondaicus*) en de Sumatraanse neushoorn (*Dicerorhinus sumatrensis*), zullen voordeel hebben bij deze verworven kennis. Alle soorten verschillen in populatie aantal alsook in mate van met uitsterven bedreigd te zijn. De zwarte, Javaanse en Sumatraanse neushoorn staan boven aan de lijst; de Indische is uiterst kwetsbaar en de witte neushoorn verontrustend laag in overgebleven aantallen.

Vermits de neushoorn in gevangenschap een verscheidenheid aan voortplantingspathologieën vertoont en zijn vruchtbaarheidsresultaten teleurstellend zijn, richt **hoofdstuk 3** zich op nieuwe inzichten in fysiologische voortplantingsparameters. In deze studie tussen 2008 en 2016 werden er 1354 witte neushoorns bestudeerd op een game-ranch van ruim 8000 hectare. De dynamische populatiegroei door zowel geboortes als aangekochte dieren werd geanalyseerd met behulp van een jaarlijkse en tweejaarlijkse groeiparameter van de kudde. In totaal werden 562 kalvingen geregistreerd. De nullipare dieren hadden een mediane leeftijd van 83.2 maanden op het moment van hun eerste kalving en hadden een gemiddelde tussenkalftijd van 29.2 maanden indien multipaar. Translocatie van de dieren had geen rechtstreekse invloed op deze parameters. Multivariate modellen tonen een seizoensgebonden geboortepatroon met pieken tussen december en april. In contrast met andere studies werd geen ongelijke geslachtsratio gevonden. Seizoensgebonden factoren of weersomstandigheden zoals regen waren niet van invloed op het geslacht van de kalveren.

Tegenwoordig wordt de stroperij tegengegaan door middel van toegenomen bewaking van reservaten alsook het preventief onthoornen van de neushoorn. De

hoorn bestaat uit keratine, vergelijkbaar met de vingernagel van de mens of de hoef van het paard. Indien op een correcte manier geknipt, bekapt of afgezaagd zal de hoorn op termijn terug groeien. **Hoofdstuk 4** focust zich, met de resultaten van 2044 onthoorningen, op de invloed hiervan op met regelmaat onthoornde individuen. Referentiewaarden en de invloed van leeftijd, aantal onthoorningen en het moment van onthoornen werden bestudeerd door middel van de groei van de voorste en de achterste hoorn (lengte, gewicht en basisomtrek) (Figuur 1). We constateerden hierbij een hogere toename in gewicht van de hoorn bij mannelijke (2.36 ± 0.082 g/dag) ten opzichte van vrouwelijke dieren (1.74 ± 0.073 g/dag). Het gemiddelde interval tussen twee onthoorningen was 587 dagen. Ook enkele dieren tuinen beslissen vandaag de dag om hun neushoorns te onthoornen vanwege veiligheidsoverwegingen. Deze studie levert daarom ook informatie op voor dieren in gevangenschap.



Figuur 1. Op de linker foto wordt de lengte van de voorste hoorn opgemeten om hem vervolgens af te zagen (rechts) op een veilige afstand van de basis (foto gemaakt door Brent Stirton, National Geographic).

In Zuid-Afrikaanse ranches worden neushoorns gehouden in verschillende kampen, enerzijds voor de fokkerij in familiegroepen, anderzijds in groepen van surplus mannetjes. Omdat mannelijke dieren in gevangenschap vroeger geslachtsrijp zijn in vergelijking tot hun wilde soortgenoten hebben wij de testosterongehalten van deze dieren gemeten en gerelateerd aan hun paargedrag. In **hoofdstuk 5** worden verschillende leeftijdsgroepen onderzocht en verbanden gezocht tussen hun

voortplanting en hun testosteronwaarde. Bij de witte neushoorn leeft het mannetje nagenoeg solitair en bezoekt deze de kudde enkel om te paren. Een significant verschil in testosteronwaarden tussen de leeftijdsgroepen kon worden aangetoond, waarbij de volwassen stieren (72 maanden en ouder) een hoger testosterongehalte hadden ten opzichte van jongvolwassenen (31-71 maanden) en jonge dieren (21-30 maanden). De dekstieren verschillen enkel significant van de stieren die niet dekken in de groep bij de adolescenten. Het aanwezig zijn van volwassen koeien draagt er mogelijk toe bij dat jonge stieren vroeger geslachtsrijp worden, hierover is echter verder onderzoek aangewezen. Neushoorns hebben een zeer zwak zicht, olfactorische prikkels kunnen daarentegen een belangrijke rol spelen in seksuele stimulatie van de dieren.

Het kweken van wilde dieren in zogenaamde wild-ranches is onderdeel van een intensieve industrie in zuidelijk Afrika. Met regelmaat uitgevoerde vruchtbaarheidsonderzoeken voor drachtdiagnose en fertiliteitsproblemen, bij zowel vrouwelijke als mannelijke dieren, kunnen hierbij een belangrijke rol spelen. Individuele dieren worden onder anesthesie gebracht voor uiteenlopende doeleinden zoals translocatie, onthoorning, identificatie en oormerken, of voor het verkrijgen van DNA monsters. **Hoofdstuk 6** toont echografisch onderzoek ter detectie van abnormaliteiten in het voortplantingsstelsel bij mannelijke (n=57) en vrouwelijke (n=63) dieren, alsook van drachtdiagnosen bij vrouwelijke dieren. In deze studie werden nauwelijks afwijkingen gevonden in contrast tot eerder gepubliceerde aantallen bij dieren in gevangenschap. In gevangenschap vertonen niet-voortplantende vrouwelijke dieren vaak zeer uitgebreide afwijkingen (cysten, gezwellen). Vervolgens werd het echografisch onderzoek van 17 vrouwelijke dieren vergeleken met fecale progesteronmetabolieten. Vroege drachtdiagnostiek, van 0-4 maanden, bleek niet betrouwbaar te zijn door middel van fecale hormoonanalyse. De progesteronwaarden bevinden zich in de orde van grootte van de luteale fase van cyclische vrouwelijke dieren. Echografisch onderzoek kan daarom waardevol zijn en routinematig worden uitgevoerd tijdens management interventies of translocaties van dieren. Daarnaast zal het ons meer informatie verschaffen over het voortplantingsstelsel van de witte neushoorn die op termijn nieuwe inzichten kan verwerven voor kunstmatige voortplantingstechnieken bij dieren in gevangenschap of zelfs in het wild.

In het laatste hoofdstuk (**hoofdstuk 7**) van dit doctoraat worden de verschillende resultaten besproken en worden er finale conclusies getrokken. Wij concludeerden dat wild ranches zeer waardevol zijn voor het verschaffen van informatie omtrent de voortplanting van de zuidelijke witte neushoorn. Niet tegenstaande de vele verschillen zijn er ook veel gelijkenissen tussen deze wild-ranches en in gevangenschap gehouden dieren. Wildparken in Zuid-Afrika worden veelal gemanaged en zijn afgebakend door omheining. Op deze plaatsen wordt steeds vaker preventief de hoorn afgezaagd en kort gehouden om de stroperij tegen te gaan en omdat vooralsnog andere maatregelen niet voldoende succesvol zijn. De studies uitgevoerd op het bedrijf in dit proefschrift kunnen daarbij dienen als leidraad. Een van de beperkingen bij onderzoek in gevangenschap is dat het aantal te bestuderen individuen vaak zeer laag is. Om de zuidelijke witte neushoorn te behoeden van uitsterven dient toekomstig onderzoek zich verder te richten op de voortplantingsfysiologie en het succesvol toepassen van voortplantingstechnieken, waarbij de vergelijking tussen wild en gevangenschap continu gemaakt dient te worden.

CURRICULUM VITAE

Cyrrillus Ververs was born on May 14, 1982 in Roermond, The Netherlands. After completing his secondary school in Roermond, VWO level, he started studying Veterinary Medicine at Ghent University, Belgium. In June 2009 he obtained the degree of Master of Veterinary Medicine.

After an internship at the ambulatory clinic of the Department of Reproduction, Obstetrics and Herd Health (DI08), Cyriel became a clinical assistant at the ambulatory clinic and was involved in day-, night-, and weekend service in the ambulatory clinic for ruminants as well as in the routine herd health visits of dairy farms. In 2012, Cyriel became an assistant at the department of Equine Reproduction (DI08) and his work included teaching, clinical service and research. His passion for wildlife led him to the start of a PhD in African white rhinoceros with the domestic horse as a model. His clinical focus now is reproduction work in equine, bovine, zoo- and wild animals with a special interest for artificial reproductive techniques (ART).

In 2016, Cyriel completed a residency in Animal Reproduction and became a European specialist (diplomate) of the European College of Animal Reproduction (ECAR).

For his PhD research, Cyriel studied southern white rhinoceroses in South Africa and was involved in several national and international wildlife reproduction project and became a member of Pro Fetura, an alliance for wildlife conservation breeding.

Cyriel (co)authored several publications in national and international peer-reviewed journals, was a speaker at multiple national and international conferences and acted as a reviewer for several scientific journals.

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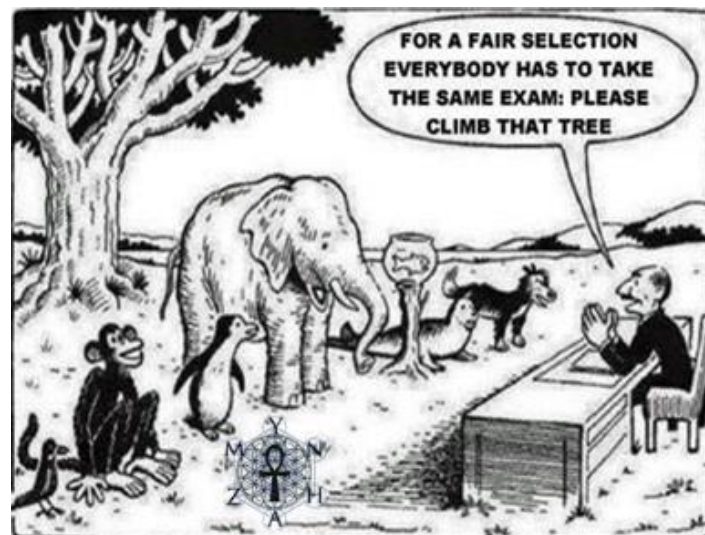
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Everybody is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid.

Albert Einstein 1879 - 1955

