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Investigating consequences of translocation-stress in pregnant black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses

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Declaration of Authenticity

I declare that the submitted thesis is original work and was written by me without further assistance. I completed the Diploma thesis independently and used only the materials that are listed. Appropriate credit has been given where reference has been made to the work of others.

All materials used, from published as well as unpublished sources, whether directly quoted or paraphrased, are duly reported.

The thesis was not examined before, nor has it been published.

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Table of Content

1 Abstract

1.1 English

Translocation and range expansion is an important strategy for white and black rhinoceros conservation to ensure effective growth rates in these near threatened and critically endangered species, respectively, by providing habitat for population growth. In order to establish viable populations, the success relies on post-release survival and reproductive efficacy.

Here, we report birth rates and birth-sex-ratios in 51 female black *(Diceros bicornis)* (BR) and 18 female white *(Ceratotherium simum)* (WR) rhinoceroses translocated between 2004 and 2019. Blood samples were collected from these animals at capture or release and serum progesterone concentrations analysed using a chemiluminescence method. Birth and sex-ratios were recorded for 18 months after translocation.

Rhinoceroses were classified as “non-pregnant” (<0.64 nmol/L, BR=5; WR=9), “luteal-activity” (>1-4.4 nmol/L, BR=17; WR=2), “3-4 months pregnant” (4.5-6 nmol/L, BR=3; WR=2), “5-8 months pregnant” (7-50 nmol/L, BR=14; WR=5), “9-12 months pregnant” (50-70 nmol/L, BR=9), and “13-16 months pregnant” (70-≥90 nmol/L, BR=3), based on their progesterone concentrations. None of the BR or WR classified as “luteal activity” and of the BR classified as “3-4-months pregnant” gave birth. Only seventeen BR, classified as “5-8-months pregnant” or higher (65 % of these classifications), gave birth to ten male (59 %), five female (29 %) and two calves of unknown sex, respectively. Post-mortem examination on one BR from this classification indicated a recent abortion. Only one WR classified as “3-4-months pregnant” (50 %) gave birth to a female calf and four WR classified as “5-8-months pregnant” (80 %) gave birth to three male and one calf of unknown sex.

Results indicate that translocation-stress might disrupt early- to mid-term pregnancies in rhinoceroses and possibly cause a sex-ratio bias towards male calves. Both observations could be related to the cortico-adrenal stress response and require further investigation.

A modified version of this abstract was presented orally at the Zoo and Wildlife Health Conference 2022 (Emmen, NL) and Anna Sickmueller was awarded the "Undergraduate Student Oral Presentation Award" as shown in Figure 1.

Figure 1: Picture of Anna Sickmueller when she was awarded the "Undergraduate Student Oral Presentation Award" at the Zoo and Wildlife Health conference 2022 (1)

INVESTIGATING CONSEQUENCES OF TRANSLOCATION-STRESS IN PREGNANT BLACK (*DICEROS BICORNIS*) AND WHITE (*CERATOTHERIUM SIMUM*) RHINOCEROSES

Anna Sickmueller, Ursina Rusch, Jacques Flamand, Henk Bertschinger, Emma Hooijberg, Leith Meyer, Kyle Burger, Markus Hofmeyr, Dave Cooper, Johanna Painer-Gigler, Friederike Pohlin

1.2 Deutsch

Untersuchung der Folgen von Translokationsstress bei trächtigen Spitzmaulnashörnern *(Diceros bicornis)* und Breitmaulnashörnern *(Ceratotherium simum)*

Translokation ist eine wichtige Maßnahme für den Nashornschutz. Die Erhaltung von Nashornpopulationen hängt vor allem von der Überlebensfähigkeit nach der Freilassung und der Reproduktionseffizienz ab.

Diese Studie untersucht die Geburtsraten und Geschlechterverhältnisse der Kälber bei 51 weiblichen Spitzmaulnashörnern (BR) und 18 weiblichen Breitmaulnashörnern (WR), die zwischen 2004 und 2019 umgesiedelt wurden. Blutproben wurden beim Einfangen oder bei der Freilassung entnommen. Die Progesteronkonzentration im Serum wurde mit einem Chemilumineszenz-Verfahren analysiert. Geburts- und Geschlechterverhältnisse wurden 18 Monate lang nach der Translokation dokumentiert.

Basierend auf ihrer Progesteronkonzentration wurden die Nashörner in fünf Klassifizierungen eingeteilt: „Nicht Trächtig“ (<0,64 nmol/L, BR=5; WR=9), „Luteale Aktivität“ (>1-4,4 nmol/L, BR=17; WR=2), „3. -4. Monat trächtig“ (4,5-6 nmol/L, BR=3; WR=2), „5.-8. Monat trächtig“ (7-50 nmol/L, BR=14; WR=5), „9.-12. Monat trächtig“ (50-70 nmol/l, BR=9) und „13.-16. Monat trächtig“ (70-≥90 nmol/l, BR=3).

Keine der als „Luteale Aktivität“ eingestuften BR oder WR und keine der als im „3. bis 4. Monat trächtig“ eingestuften BR gebaren. Lediglich 17 von 24 BR, die als im „5.–8. Monat trächtig“ oder höher eingestuft wurden (65 % dieser Klassifizierungen) gebaren. Diese brachten zehn männliche (59 %), fünf weibliche (29 %) und zwei Kälber unbekannten Geschlechts zur Welt. Die Obduktion bei einem BR dieser Klassifikation ergab einen Abort. Nur eine als „3.-4. Monat trächtig“ eingestufte WR (50 %) brachte ein weibliches Kalb zur Welt und vier als „5.-8. Monat trächtig“ eingestufte WR (80 %) brachten drei männliche und ein Kalb unbekannten Geschlechts zur Welt.

Die Ergebnisse zeigen, dass Translokation frühe und mittlere Trächtigkeitsstadien bei Nashörnern gefährdet und möglicherweise eine Verzerrung des Geschlechterverhältnisses zugunsten männlicher Kälber verursacht. Beide Beobachtungen könnten mit der kortiko-adrenalen Stressreaktion zusammenhängen und erfordern weitere Untersuchungen.

Eine abgewandelte Version dieses Abstrakt wurde an der Zoo and Wildlife Health Conference 2022 (Emmen, NL) präsentiert. Anna Sickmueller gewann hierbei den "Undergraduate Student Oral Presentation Award".

INVESTIGATING CONSEQUENCES OF TRANSLOCATION-STRESS IN PREGNANT BLACK (*DICEROS BICORNIS*) AND WHITE (*CERATOTHERIUM SIMUM*) RHINOCEROSES

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2 Abbreviations

Abbreviation	Description
ACTH	Adrenocorticotrophin Hormone
APR	Acute Phase Reaction
BR	Black Rhinoceros
BRREP	Black Rhino Range Expansion Project
BSR	Birth Sex Ratio
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
EKZNW	Ezemvelo KwaZulu Natal Wildlife
HPA	Hypothalamic–Pituitary–Adrenal
IUCN	International Union for Conservation of Nature and Natural Resources
LH	Luteinizing Hormone
OS	Oxidative Stress
RWB	Rhinos Without Borders
SA	South Africa
TWH	Trivers-Willard Hypothesis
WR	White Rhinoceros
WWF	World Wildlife Fund

3 Introduction

3.1 Threatened Species

Rhinoceroses, or rhinos, once roamed in great numbers of up to 500,000 individuals at the beginning of the 20th century throughout Eurasia and Africa. They were part of one of the five extant species of odd-toed ungulates in the family *Rhinocerotidae*. They are one of the largest remaining megafaunas and today only 27,000 remain. Three rhino species, the greater one-horned Indian rhinoceros (*Rhinoceros unicornis*), the Javan rhinoceros (*Rhinoceros sondaicus*) and the Sumatran rhinoceros (*Dicerorhinus sumatrensis*), are native to Asia. Whereas the two rhino species covered in this thesis, the white rhinoceros (*Ceratotherium simum*) and the black rhinoceros (*Diceros bicornis*), inhabit the African continent. The white rhino is the most common subspecies of the family *Rhinocerotidae* consisting of 17,212–18,915 free ranging mature individuals, followed by the black rhino with 5,366 to 5,627 mature individuals in 2017 (2).

Rhinos are often referred to as “flagship species”, which means they are a charismatic species that can act as an ambassador to raise support or money for conservation projects or environmental causes. They attract donors and ecotourism, are highly likeable and easy to advertise with, and are of high national or even spiritual value, which brings additional benefit to their conservation (3). The introduction of rhinos into a new protected area often also facilitates the introduction of other IUCN red listed species such as lions, pangolins and wild dogs.

As an overview, the white rhino is listed as “Near Threatened” by the International Union for Conservation of Nature and Natural Resources’ (IUCN) Red List of Threatened Species. Despite white rhino numbers declining rapidly, they do not reach critical thresholds to be assessed as “Vulnerable”. Neither can they be listed as “Least Concern”, even though protection efforts have helped stabilise the numbers. Yet, without continuing conservation efforts numbers could decline significantly due to the continued high levels of poaching and increased involvement of organized international criminal syndicates associated with the illegal international rhino horn trade (4).

Black rhino numbers have doubled since the 1990s due to increased protection and population management by creating more active subpopulations. However, they are still listed as “Critically Endangered” due to the scale of the decline in numbers over the past three generations caused by skyrocketing levels of illegal poaching (5).

3.2 Translocations

Since the early sixties rhino translocations have become a critical tool for the protection of these endangered animals (3). Several rhino range states started working together and took action to translocate rhinos to secure regions within their former range (6).

Translocation is defined as the deliberate human-mediated movement of individuals or populations from one site for release in another suitable location with the intention of a measurable conservation benefit at the levels of a population, species, or ecosystem (7).

The objective in rhino translocation is to expand the rhino's range and to stimulate growth rates in both the source- and newly established populations with the incentive to strengthen the numbers as quickly as possible. New source populations are necessary to preserve genetic diversity in the long term, and to provide the biggest possible buffer against future potential poaching losses (6).

The need for conservation and protection strategies, including species management interventions such as translocation, reintroduction, and reinforcement programs for metapopulation management, is ongoing. Thus, translocation symbolises an essential tool for the management of these species, and it remains an integral part of national and international rhino conservation plans (8).

The modelled projected trends of the IUCN of African rhino numbers into the future as part of Red List assessments rely heavily on translocation projects and other conservation measures such as monitoring, field rangers, anti-poaching units, dehorning projects, and others (2).

3.3 Stress Response

With the increasing numbers of conservation interventions, such as translocations, it is vital to improve the success of those operations. Rhino translocation is associated with stress responses, which, if understood can be considered a predictable factor and can be incorporated into planning (9). Therefore, it needs to be assessed how acute and chronic stress affect the outcomes of conservation programs and contribute to morbidity and mortality. Translocation failure can result from indirect effects of different stressors, by increasing the vulnerability of the individuals, and decreasing the sustainability of the projects (9).

The stress response is a physiological response in animals to stressors. Two response chains can be activated and need to be distinguished: acute and chronic stress (9).

The acute stress response is fast acting and mediated by the sympathetic nervous system, and the release of the catecholamines, epinephrine and norepinephrine. Initiation of the fight-or-flight response allows for a quick reaction to the stressor and includes elevation in heart rate, increased blood pressure, and mobilization of energy sources to the central nervous system and somatic muscle (9).

The acute stress response is then followed by the slower hormonal stress response, in which the adrenal gland secretes glucocorticoid hormones, such as cortisol. The hormone production is the result of a hormonal cascade along the hypothalamic-pituitary–adrenal axis. It begins with stimulation of the hypothalamus, which signals the pituitary to secrete adrenocorticotrophin hormone (ACTH), which in turn stimulates the adrenal gland to release glucocorticoids. Negative feedback quickly suppresses glucocorticoid release, if this is not the case, stress can become chronic (10).

Translocation includes both an acute and chronic stress response. The variety of stressors the animals are exposed to during and after translocations include, but are not limited to capture, immobilization, temporary captivity, transport, and release into a novel environment. The process can also lead to prolonged periods of water- and food deprivation (11).

Rhinos have been shown to exhibit a stress response to capture and transport characterized by an increase in plasma epinephrine and serum cortisol concentrations, stress-hemoconcentration, an increase in neutrophil to lymphocyte ratio, an acute phase reaction (APR) and oxidative stress (OS) (12). Rhinos transported over a long distance additionally experienced dehydration, a negative energy balance, skeletal muscle fatigue, and stress-induced immunomodulation (13).

There are several consequences associated with the repetitive or prolonged exposure to stressors, such as during translocation. The initial short-term stress is meant to serve a fast response. It initiates immune responses, which serve a protective purpose and are a fundamental survival mechanism in an acute situation by preparing the immune system for challenges (14).

However, in chronic situations, where these responses persist from days to months, they become suppressed or dysregulated, which leads to pathological outcomes (14). Chronic stress effects are tied to factors that have been directly linked to translocation failure such as: starvation, increased susceptibility to disease, predation, dispersal and, most importantly to decreased reproductive capacity and reproductive failure (9).

3.4 Rhino Pregnancies

3.4.1. Reproductive physiology

In the wild, rhinos are described as polyestric, non-seasonal breeders. Oestrous cycles in rhinos are quite diverse. Oestrogen and progesterone profiles measured in the feces, urine and serum using various hormone assays characterized the oestrous cycle with different cycle lengths in each species: 27 days in the black and 30–35 or 65–70 days in the white rhino (15,16).

Ovulation in the rhinos is induced by a single, pre-ovulatory luteinizing hormone (LH) surge that occurs at the end of the follicular phase (15).

The gestation period of rhinos is the second longest in terrestrial mammals ranging from 15 to 18 months.

Pregnancy can be diagnosed by elevated progesterone concentrations 3–5 months (17,18) after conception, or by ultrasound 2–4 weeks post-breeding. The embryonic vesicle is detectable by ultrasound as early as 15 days post-ovulation (15,19).

Progesterone is produced in the corpus luteum as well as in the placenta and prepares and maintains the endometrium to allow implantation and helps to maintain a healthy pregnancy. Progesterone is also important in suppressing the maternal immunologic response to fetal antigens, thereby preventing maternal rejection of the trophoblast (19,20).

Oestrogens remain at low levels throughout most of gestation and, therefore, do not appear to be useful indicators of pregnancy or foetal health in the black rhino (17,21).

Furthermore, no chorionic gonadotrophin has been identified in rhinos, but it is possible that rhinos produce early-pregnancy proteins, like relaxin, as do most other species, but more research needs to be carried out (17,22).

3.4.2. Disruption of Pregnancies

There is only limited information regarding the disruption of pregnancies in wild rhinos, however early embryo loss has been reported in captive black and white rhinos (17).

Rhinos are closely related to horses as they too belong to the order *Perissodactyla, which* includes three families: horses (*Equidae*), rhinoceroses (*Rhinocerotidae*), and tapirs (*Tapiridae)*. All perissodactyls are odd-toed ungulates, hindgut-fermenting herbivores and include grazers, browsers, and mixed feeders (23), which is why information regarding their physiology is often used interchangeably when species-specific information is lacking.

Stress is known to have a major impact on pregnancies in horses and other mammals. Stress does not only trigger premature foaling but also prolongs the expulsion rate of foals during parturition, which subsequently increases the cortisol concentration in equine and bovine neonates significantly in the immediate postnatal period (24).

In horses, the stress associated with colic surgery was found to influence foaling rates depending on the timing of gestation. Early pregnancies (<40 days) had a lower foaling rate than those with 40 days or higher, indicating that embryos are more susceptible to hormonal changes at earlier stages. Likewise, the foaling rate of the mare decreased significantly with higher age of the mare. The duration of anesthesia also had a negative effect on the foaling rate, while hypotension, hypoxemia and post-operative endotoxemia did not influence it (25).

That there is a connection between stressful events and pregnancy loss in mammals has already been described decades ago. Stressful conditions, like transportation, surgery, pain etc. can depress the progesterone output by the primary corpus luteum, which may be mediated by cortisol, resulting in embryonic or fetal loss (26).

Early embryo loss in captive rhinos has been associated with conditions like endometritis, pyometra, leiomyoma and luteal insufficiency which describes a deficiency by the corpus luteum in progesterone production (27). Stress is often high in captive animals and is mentioned as a cause for acyclicity (28).

In the wild, natural calf mortality caused by, for example predation or fights in black rhinos, in the first year after birth is high, ranging between 8 % and 14 % (29).

Little is known regarding the effect of translocation on rhino pregnancy and calving rates.

In rhinos, as well as in other species, such as horses, long lasting transports result in OS, an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to oxidative damage (13,30). OS is involved in the initiation or development of pathological processes in reproduction, such as embryonic resorption, recurrent pregnancy loss, preeclampsia, and intrauterine growth restriction could lead to fetal death in these species (31).

So far, in transported rhinos, poor calf survival rather than early abortion has been observed. Brett (1998) reported the disruption of calves born within a year after their mother’s translocation. He noted that there was poor survivorship of calves whose mothers were translocated while pregnant, hereby implying that poor survival rate is due to translocation stress in the mother. There was only a 50 % chance of surviving the first year, while 3 out of 4 deceased calves died in the first week after birth (32).

3.5 Sex-Ratio Bias

Apart from disrupted pregnancies, captive rhinos face another problem. A sex ratio skewed in favour of male calves has been reported to pose an additional challenge to wildlife managers (28,33).

Birth sex ratios (BSR) are a highly discussed topic with varying results depending highly on the species reproductive strategy, social structure, and environmental framework. The following chapters provide a comprehensive insight into several important theories.

3.5.1 In Other Animals

One of the oldest hypotheses concerning BSR in mammals is the so-called Trivers-Willard hypothesis (TWH) (34). It suggests that a male in good condition is expected to have a higher reproduction rate than a sister in the same condition, while she is expected to out-reproduce him if both are in poor condition. This means that natural selection would favour the ability of the parent to adjust the sex of the offspring according to their current investment capacity (34).

Data from several mammalian species support this model: As maternal condition declines, the adult female tends to produce a lower ratio of males to females (34).

Yet empirical studies produce conflicting results. One species in which the TWH is not applicable is the American bison (*Bison bison*). There was little evidence that mothers in better condition bore more sons. Maternal condition was barely related to offspring sex ratio, although the last calves of old females were nearly always female (35).

As a consequence, attempts have been made to reformulate the TWH aiming at presenting models that predict optimal comprehensive multielement parental investment strategies beyond the classical TWH and incorporate more influencing factors (36).

Such factors include sex differences in energy requirement, viability during early growth, relative fitness, and competition or cooperation between siblings and/-or parents (37).

Parental fitness and investment both have a great effect on the breeding success of one sex. In red deer *(Cervus elaphus)* and rhesus macaques *(Macaca mulatta)*, the sons of the dominant mothers with greater reproductive performance and superior condition are more successful than their daughters. Dominant mothers are also more likely to give birth to sons and show higher investment towards them. In subordinate mothers, the sex ratio is skewed towards female offspring, which are more successful than their sons (37).

In mammals exposed to environmental stress, several sex allocation mechanisms that can cause a sex bias in the offspring have been proposed (38):

- Hormone-induced conception bias,
- Sex-differential embryo death from excess glucose metabolism,
- Sex-differential embryo death from embryo–uterine developmental asynchrony,
- Pregnancy hormone suppression and resource deprivation.

All these above can be switched on by the cortico-adrenal stress response (38).

3.5.2 In Rhinoceros

Studies on rhinos indicate a biased BSR under different conditions.

Translocation-stress induced a statistically significant BSR of 86 % male births in early gestation and only 38 % male in mid-gestation. Moreover, the authors found a strongly male-biased 67 % BSR for conceptions after translocation and arrival in captivity (38).

Furthermore, there appears to be a sex bias towards male calves in the wet season, where there is generally a more favourable environment and better nutrition available than in the dry season. A recent study found a BSR of 57 % male births in wet seasons, in accordance with the TWH. Plus, mothers were more likely to raise male calves when conceived during wet years with a percentage of 60 % (39).

A previous study investigated one of the founder populations of the black rhinos from this study. They found no significant differences in the sex ratio of black rhino calves born between 1998 and 2013 in the “Hluhluwe and iMfolozi Game Reserve”, which is located in KwaZulu-Natal, South Africa (SA) (40).

3.6 Study Objectives and Hypothesis

Translocation is a critical conservation strategy and crucial for rhino conservation, but to establish viable populations, translocation success relies heavily on post-release survival and reproductive efficacy (3). It is therefore important to understand the consequences translocation-stress might have on pregnancy and calving rates in rhinos.

The present study aimed to investigate consequences of translocation stress including the loss of calves and differences in BSR in black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinos of different pregnancy stages, and the effect of capture and long road transport on pregnancies.

Hypothesis 1: The stress associated with capture and transport disrupts pregnancies in black and white rhinos.

Hypothesis 2: The stress associated with capture and transport affects the BSR in black and white rhinos.

4 Material and Methods

4.1 Ethical Permit

Routine post-translocation observational monitoring data collected from 51 female black and 18 female white rhinos translocated between 2004 and 2019 as part of two rhino conservation projects unrelated to this study were used for this retrospective analyses.

One project is the “Black Rhino Range Expansion Project” (BRREP) of the World Wildlife Fund (WWF) (41), the other is the “Rhinos Without Borders” (RWB) Project (42).

Blood sample collection was performed from all rhinos for pregnancy assessment (progesterone concentrations) unrelated to this study. Some blood samples were analysed more extensively and included in a study by Pohlin et al. (2020) (13). The University of Pretoria Animal Ethics Committee approved the collection of these blood samples from these rhinos and the subsequent analysis (V067-17) (13).

4.2 Background and Study Animals

4.2.1 Black rhino range expansion project

BRREP’s main aim is to expand the black rhino’s range and to increase growth rates in this critically endangered species (41). The Logo can be seen in Figure 2.

Figure 2: Picture of the logo of the black rhino range expansion project (BRREP) and World Wide Fund for Nature (WWF) (41)

BRREP was founded in 2003 by the wildlife veterinarian and former state veterinarian in the Kruger in the 70's, Dr. Jacques Flamand. He was also the veterinarian for the Ezemvelo KwaZulu Natal Wildlife (EKZNW) the KwaZulu-Natal provincial conservation agency for nearly 20 years (43).

EKZNW realized in the early 2000's that their black rhino population growth rates were levelling off. They realized that this observation was likely caused by the populations reaching carrying capacity, which is why female rhinos increased their inter-calving intervals. To alleviate this problem, 5 % of the populations per annum are harvested and BRREP finds new habitat for these rhinos. They are not only placed into reserves around SA, but also reintroduced to other countries such as Malawi. Additional to the population in Liwonde National Park in Malawi, there are currently a total of 13 BRREP populations around SA (41,43).

4.2.2 Study animals from BRREP

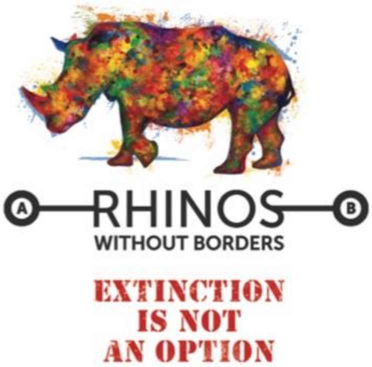
From the BRREP Translocations, serum progesterone concentrations and post-release calving rates from following rhinos were provided for this study:

- 7 female black rhinos, who were boma-adapted and from the KwaZulu-Natal founder population, were brought to a private game reserve release site in the north of SA in 2017.
- 9 female black rhinos released in the Buffalokloof population in 2019 in SA.
- 12 female black rhinos released in the Liwonde population in Malawi in 2019.
- 23 female black rhinos, historical data from 2004 to 2017, different founder and receiver populations. This group is the only one where serum progesterone levels were not tested in all individuals. Instead, there was an estimated month of pregnancy at transport based on the calving records and, instead of exact progesterone measurements, we have access to the pregnancy categories as described in Figure 7.

4.2.3 Rhinos without borders

Rhinos without borders is a joint initiative between "andBeyond" and "Great Plains Conservation", two leading conservation and tourism companies. The Logo can be seen in Figure 3. Its objective is to translocate rhinos from high poaching risk areas in SA to create new source populations in Botswana's wilderness areas, as visualized in Figure 4. where only isolated poaching incidents had been reported at that time (42).

Figure 3: Picture of the Logo of Rhinos Without Borders (42)



The initial success of this project was evident not only in the total of over 130 animals moved to those areas, but also given the fact that every sexually mature female that was translocated has had a calf (42). However, due to ill-advised decision making of president Mokgweetsi Masisi in recent years, Botswana anti-poaching units have been disarmed and the country is currently experiencing rising cases of rhino poaching. At least 100 animals have been killed over the past three years and the Department of Wildlife and National parks has taken a decision to relocate the remaining animals to an unspecified location in the Northwest District of Botswana to protect them from poachers (44).

Figure 4: Description of the Rhinos Without Borders Project, showing a map of Africa and the countries to and from which the rhinos were translocated with additional information regarding the former poaching status (42)

4.2.4 Study animals from Rhinos without borders

From the RWB Translocations, serum progesterone concentrations and post-release calving rates from following rhinos were provided for this study:

- 18 female white rhinos, originating from a game-farm in SA, that were transported just over 1,300 km to Botswana in 2017.

4.3 Capture and Transport

BRREP is most famously known for their "flying rhinos", where black rhinos that are in inaccessible areas are darted and flown out with a helicopter, as seen in Figure 5. Selected rhinos are darted, then slung out upside down by their feet and transported to locations, that are more easily accessible for the team. Then they are loaded into crates, onto trucks and either driven directly to their new home or first housed in bomas for quarantine and then moved to their new home. These translocations happen once every year or two (41).

Figure 5: Picture showing the black rhino range expansion project's (BRREP's) famous flying rhinos (43)

In 2019, rhinos were moved to Liwonde in Malawi by crating them, loading them onto a Boeing 747 out of Durban and flying them to Malawi. Three of the females were airlifted by helicopter at capture, the remainder were transported via road only.

The Buffalokloof population was also established in 2019. The reserve is in the Eastern Cape province and the rhinos were moved within the province from a provincial reserve called Great Fish Nature Reserve to Buffalokloof. Great Fish is highly inaccessible, so all rhinos moved were darted, slung out by helicopter, and then transported by road to Buffalokloof (41).

The seven black rhinos in the first BRREP group were boma-adapted and transported by road just over 600 km from KwaZulu-Natal to the north of SA in 2017 (13). The 18 female white rhinos from RWB Project were transported just over 1,300 km from SA to Botswana in 2017. Detailed information about these two translocations, the drugs administered, and samples collected can be found in Pohlin et al. (2020) (13).

Briefly, the BR were only sedated via darting by foot or helicopter with a combination of 0.6–0.8 mg (low-dose) etorphine (Captivon, 9.8 mg/mL, Wildlife Pharmaceuticals, Karino, SA), 60 mg azaperone (Azaperone tartrate, 50 mg/mL, Wildlife Pharmaceuticals) and 5000 IU hyaluronidase delivered remotely using 1.5-mL plastic darts (DAN-INJECT, International S.A., Skukuza, SA) with 60-mm uncollared needles propelled by compressed air. With this combination BR were directly loaded into the crate where they received 1.2 mg diprenorphine (Activon, 12 mg/mL, Wildlife Pharmaceuticals) and 10 mg butorphanol (Butorphanol tartrate, 50 mg/mL, Wildlife Pharmaceuticals) intravenously and 1 g carprofen (Rimadyl, 50 mg/mL, Zoetis, Sandton, SA) intramuscularly. The WR were fully immobilized by darting from a helicopter with a combination of 3–5 mg etorphine, 20– 40 mg azaperone, and 5,000 IU hyaluronidase (Hyalase, Kyron Laboratories, Johannesburg, SA) delivered remotely using 2.0-mL darts (Pneu-dart, Inc.t, Williamsport, Pennsylvania, USA) with 63.5-mm barbed needles (13). Within 10 minutes of darting, 0.2–0.8 mg diprenorphine (M5050, 12 mg/mL, Novartis, Midrand, SA) was administered intravenously to partially reverse the immobilizing effects of the etorphine and load the WR into the transport crate, where another 2.5–15 mg of intravenous diprenorphine were administered to complete the etorphine reversal. The tranquilizer zuclopenthixol acetate (Clopixol-Acuphase, 50 mg/mL, H. Lundbeck Pty. Ltd., Randburg, SA) was administered intramuscularly via hand-injection to all rhinos of both translocations (150–220 mg/BR or 100–250 mg/WR) just after loading into the crates (13).

Only International Air Transport Association-approved rhino crates were used in all translocations, which followed the practical guidelines for transport of live wild animals (45) and rhinos (46). Transport commenced once all rhinos had been captured and loaded, which took a mean of 3.25 ± 0.75 SD hours in both translocations. During transport, the vehicles stopped at 2–4-h intervals to allow for additional intramuscular administration of azaperone and, or midazolam (Dazonil, 50 mg/mL, Wildlife Pharmaceuticals) to restless animals. At the release site, rhinos were re-immobilized with etorphine (3.5–4 mg/BR or 3.5–6 mg/WR) and azaperone (40 mg/BR or 20–40 mg/WR) via pole syringe or hand injection into the nuchal hump while standing in the transport crate. Before animals became immobile, they were released from their crates and manually restrained with ropes until they became recumbent. After sample collection, marking and collaring of the animals, naltrexone (Trexonil, 50 mg/mL, Wildlife Pharmaceuticals), at 20 times the etorphine dose in mg, was administered intravenously to reverse the immobilization and release the rhinos into the private game reserve (BR) or national park (WR) (13).

4.4 Sample Collection and Analysis

Venous blood samples collected at capture from the RWB Project rhinos and from the seven BRREP rhinos translocated in 2017 was collected directly into serum and potassium oxalate/sodium fluoride (NaF) tubes (BD Vacutainer, Becton and Dickinson, Plymouth, UK), stored in a cooler box with ice packs, and centrifuged within 24 h. Serum and plasma were aliquoted and stored at -80 °C until analysis. Samples from the white rhinos were stored at -20 °C for 1 month prior to being stored at -80 °C (13).

Samples from the other BRREP translocations strictly followed their in-house developed protocol, generously made available by Prof. Henk Bertschinger as seen in Figure 6.Blood samples were analyzed in the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital, University of Pretoria, SA.

An assay methodology for measuring the serum progesterone concentrations following chemiluminescence method system was employed: before 2018 the “Immulite ® 1000” (Immunoassay Systems by Siemens Healthcare Diagnostics) was used, after 2018 it was replaced by the “Immulite ® 2000” (Immunoassay Systems by Siemens Healthcare Diagnostics).

Birth and sex-ratios of calves at the release sites were recorded for 18 months after translocation by active monitoring for conservation purposes (47).

Figure 6: Protocol for rhino samples from the black rhino range expansion project (BRREP) by Prof. Henk Bertschinger, describing how serum samples are being collected and how to not handle the samples including the

Protocol for Rhino Samples

Serum samples

- Collect blood in 10 ml plain red-topped venoject tube – they do not contain anticoagulants or gel accelerators/separators
- Leave at room temp (>25 C°) for 30-60 min
- Place on ice for up to 3 h
- Separate serum preferably by centrifugation.
- Transfer serum to plastic vials or unused clean plain venoject tubes and store at minus 18-20 C°
- Ship on ice

Alternative in case of emergency
If you do not have plain venoject tubes, you can use EDTA or heparinised tubes.

- Place immediately on ice
- Centrifuge as soon as possible
- Separate plasma by centrifugation.
- Transfer plasma to plastic vials or unused clean plain venoject tubes and store at minus 18-20 C°
- Ship on ice

DO NOT

- **Collect samples in clot-accelerator and gel separator tubes – affects progesterone concentrations as gel binds progesterone**
- **Do not freeze plain or EDTA/heparinised blood tubes prior to removing the serum. Haemolysis affects accuracy of the assay.**
- **Do not send whole blood samples – causes haemolysis and progesterone metabolised by blood cells**

4.5 Data and Statistical Analysis

Included in this study were a total of 69 rhinos, consisting of 51 female black rhinos and 18 female white rhinos. For all of them, the observed birth rates and birth-sex-ratio of their calves were recorded. Serum progesterone concentrations and resulting pregnancy classifications (Figure 7) were available in 46 rhinos (Table 1). In the remaining 23 black rhinos, estimated pregnancy at transport was back-calculated based on when the calf was born, on the basis of a pregnancy duration of 16 months (n=14); or the serum progesterone concentrations were measured, but instead of the exact serum progesterone concentration results, only the pregnancy classification of these animals was made available to us (n=14) (Table 2). In five individuals, estimated pregnancy at transport as well as pregnancy classification based on progesterone concentration were available. To conclude, there were nine individuals where only estimated pregnancy by back-calculation was available.

Thus, rhino pregnancies were either classified based on their serum progesterone concentrations (n=53) or their estimated duration of pregnancy (n=14). The serum progesterone concentrations used to classify rhino pregnancy are currently being validated in a study by Prof. Henk Bertschinger, a wildlife reproductive expert of the University of Pretoria. He generously made his current preliminary results of pregnancy classification based on serum progesterone concentrations available for use in this study (Figure 7).

Figure 7: Pregnancy classification and considerations based on serum progesterone concentrations in rhinos made available by Prof. Henk Bertschinger, currently being validated

Progesterone categories and comments used in reports by Prof. Henk Bertschinger

Approximate concentrations at various stages of pregnancy are:

- 3-4 months pregnant: 4.5-6 nmol/L
- 5-8 months pregnant: 7-50 nmol/L
- 9-12 months pregnant: 50-70 nmol/L
- 13-16 months pregnant: 70-90 (and greater) nmol/l

Note: False positive results may be found in the presence of a persistent corpus luteum and pyometra.

Note: False negative concentrations may be found very early in pregnancy or during last 2 weeks of pregnancy.

Cows with baseline progesterone concentration (<0,64 nmol/L) that is not compatible with a pregnancy.

Cows that have evidence of luteal activity, but it is below the cut-off concentration for pregnancy (>1-4.4 nmol/L) in rhino. They may have a cyclic CL or be early or very late pregnant.

The data were assembled and merged, and, as shown in Table 1 and Table 2, the classifications of both serum progesterone concentrations and estimated pregnancy month were adjusted to one another.

Table 1: Table containing female rhino ID, rhino species, serum progesterone concentrations, resulting pregnancy classification at translocation and calf results

No	Rhino ID	Species	Progesterone [mmol/l]	Pregnancy classification	Calf
1	RWB - 1	WR	0.668	Luteal activity	No
2	RWB - 4	WR	16.9	5-8 months	U
3	RWB - 5	WR	5.31	3-4 months	No
4	RWB - 6	WR	<0.64	Non-pregnant	-
5	RWB - 10	WR	5.98	3-4 months	F
6	RWB - 12	WR	25.7	5-8 months	M
7	RWB - 13	WR	1.10	Luteal activity	No
8	RWB - 14	WR	<0.64	Non-pregnant	-
9	RWB - 15	WR	25.7	5-8 months	M
10	RWB - 17	WR	<0.64	Non-pregnant	-
11	RWB - 18	WR	<0.64	Non-pregnant	-
12	RWB - 19	WR	36.6	5-8 months	M
13	RWB - 21	WR	<0.64	Non-pregnant	-
14	RWB - 23	WR	<0.64	Non-pregnant	-
15	RWB - 24	WR	<0.64	Non-pregnant	-
16	RWB - 26	WR	<0.64	Non-pregnant	-
17	RWB - 30	WR	9.70	5-8 months	N
18	RWB - 32	WR	<0.64	Non-pregnant	-
19	WWF17 - Tube 1 - BF001	BR	4.3	Luteal activity	No
20	WWF17 - Tube 3 - NN135	BR	<0.64	Non-pregnant	-
21	WWF17 - Tube 6 - NN56	BR	65.5	9-12 months	M
22	WWF17 - Tube 9 - 136/16	BR	81.4	13-16 months	M
23	WWF17 - Tube 10 - 125/16	BR	<0.64	Non-pregnant	-
24	WWF17 - Tube 11 - 133/16	BR	0.7	Luteal activity	No
25	WWF17 - Tube 13 - 130/16	BR	0.7	Luteal activity	No
26	Buffalokloof 19 - Hlobo	BR	0.67	Luteal activity	No
27	Buffalokloof 19 - Diza	BR	1.25	Luteal activity	No
28	Buffalokloof 19 - Londoloza	BR	3.1	Luteal activity	No
29	Buffalokloof 19 - Onwabile	BR	7.38	5-8 months	No

30	Buffalokloof 19 - Sixto	BR	14	5-8 months	No
31	Buffalokloof 19 - Nonophela	BR	1.25	Luteal activity	No
32	Buffalokloof 19 - Dave	BR	4.2	Luteal activity	No
33	Buffalokloof 19 - Ncuma	BR	7.28	5-8 months	No
34	Buffalokloof 19 - Hilda	BR	4.99	3-4 months	No
35	Liwonde - 11/19	BR	28.3	5-8 months	No
36	Liwonde - 12/19	BR	<0.64	Non-pregnant	-
37	Liwonde - 13/19	BR	<0.64	Non-pregnant	-
38	Liwonde - 14/19	BR	<0.64	Non-pregnant	-
39	Liwonde - 17/19	BR	38.5	5-8 months	F
40	Liwonde - 18/19	BR	25.6	5-8 months	No
41	Liwonde - 19/19	BR	1.3	Luteal activity	No
42	Liwonde - 26/19	BR	1.63	Luteal activity	No
43	Liwonde - 28/19	BR	2.47	Luteal activity	No
44	Liwonde - 30/19	BR	27.5	5-8 months	Abort
45	Liwonde - 31/19	BR	1.36	Luteal activity	No
46	Liwonde - 32/19	BR	42.3	5-8 months	No

Table 2: Table containing female rhino ID, rhino species, pregnancy classification based on serum progesterone concentration, estimated pregnancy months at translocation based on back-calculation from birth, pregnancy classification based on either serum progesterone concentration or estimated pregnancy months, and calf results

No	Rhino ID	Species	Progesterone concentration	Calculated months	Pregnancy classification	Calf
47	BF251	BR	4 to 5	6	5-8 months	F
48	BF34	BR	-	14	13-16 months	M
49	BF-306-05	BR	-	9	9-12 months	M
50	BF-270-05	BR	-	8	5-8 months	F
51	BF32	BR	12 to 14	10	9-12 months	M
52	BF38	BR	5 to 8	10	9-12 months	M
53	TBF70	BR	-	11	9-12 months	M
54	TBF72	BR	-	10	9-12 months	F
55	SRBF01	BR	-	10	9-12 months	F
56	BR-F4	BR	-	7	5-8 months	M

57	BR-F2	BR	-	9	9-12 months	U
58	BF02	BR	-	14	13-16 months	U
59	BF154	BR	9 to 12	11	9-12 months	M
60	BF04	BR	13 to 15	7	5-8 months	M
61	BF170	BR	early or cyclic CL	-	Luteal activity	No
62	BF01	BR	early or cyclic CL	-	Luteal activity	No
63	BF14	BR	4 months or cyclic CL	-	3-4 months	No
64	BF-02-05	BR	3 to 4	-	3-4 months	No
65	BF-122-05	BR	5 to 8	-	5-8 months	No
66	BF26	BR	5 to 8	-	5-8 months	No
67	BF304	BR	early or cyclic CL	-	Luteal activity	No
68	BF333	BR	early or cyclic CL	-	Luteal activity	No
69	BF00	BR	early or cyclic CL	-	Luteal activity	No

In the analysis of the data for possible disrupted pregnancies, rhinos who showed luteal activity but were below the cut-off concentration for pregnancy (>1-4.4 nmol/L) were excluded. These animals may have a cyclic corpus luteum or are at early or very late pregnancy. Out of 69 rhinos, a total of 36 animals met our criteria, consisting of 29 BR and 7 WR that were included in further analysis.

Descriptive analyses of results included the investigation whether rhinos classified as 3 months pregnant or higher actually gave birth or if pregnancy was disrupted. Descriptive tables and diagrams were generated using EXCEL in order to visualize pregnancy classifications and rhino births and to calculate the percentage of disrupted pregnancies and births. In a second step, the sex of the new-born calves was visualized and BSR calculated and visualized using tables and diagrams.

Additionally, odds ratios were calculated using MedCalc (www.medcalc.org) to investigate whether:

1) Early- to mid- term pregnancies (3-8 months) in comparison later pregnancies (9-16 months) had a higher chance of possible pregnancy loss
2) Calves born from transported rhinos had a higher chance of being male compared to calves from the founder population. The compared numbers were derived from Nhleko

et al (2017) from one of the founder populations in the Hluhluwe–iMfolozi Park, KwaZulu-Natal that consisted of 67 females in 2013 and demonstrated a birth rate of 0.3 and a sex ratio of 1:1 male:female in calves (40). This means that, calculating 67 female rhinos with a birth rate of 0.3 are approximately 20 births per year, of whom 10 would be male calves and 10 female calves. Thus, male (n=13) and female (n=6) calves of transported rhinos were compared against determined male (n=10) and female (n=10) calves of non-transported rhinos from the founder population.

3) There was a BSR bias of male vs. female calves resulting from rhinos translocated during early (3-8 months) vs mid-term (9-16 months) pregnancy.

Table 3 shows the percentages and numbers, including a statistical correction of 0.5 of the individual black and white rhinos with or without possible pregnancy loss combined in only two groups of pregnancy classification (3-9 months and 9-16 months). We again excluded rhinos that were not pregnant or only showed luteal activity.

To circumvent the zero-cell problem the modified Haldane-Anscombe correction was used. It is defined as one may add 0.5 to all cells of the 2x2 table only if there is at least one zero (48).

The level of statistical significance was set at P = 0.05.

Table 3: 2x2 table used for Odds ratio calculation, showing two pregnancy classifications, early- to mid-term and late pregnancy with the percentages and number of rhinos who were either pregnant or not pregnant

	3-8 months		**9-16 months**	
	%	**Number of individuals**	**%**	**Number of individuals**
Possible Pregnancy Loss	58.3 %	14	0.0 %	0
Calf born	41.7 %	10	100.0 %	12

5 Results

Results are summarized in Table 4.

Table 4: Summary of results showing all pregnancy classifications, the number of black and white rhinos in them and the number and sex of the calves

Pregnancy classification	Black rhino			White rhino		
	n rhino	n calves	sex	n rhino	n calves	sex
Non-pregnant	5	-	-	9	-	-
Luteal activity	17	-	-	2	-	-
3-4 months pregnant	3	0	-	2	1	1 ♀
5- 8 months	14	5	3♀ 2♂	5	4	3 ♂ 1u
9-12 months	9	8	2♀ 6♂ 1u	0	-	-
13-16 months	3	3	2♂ 1u	0	-	-

5.1 Rhino pregnancies

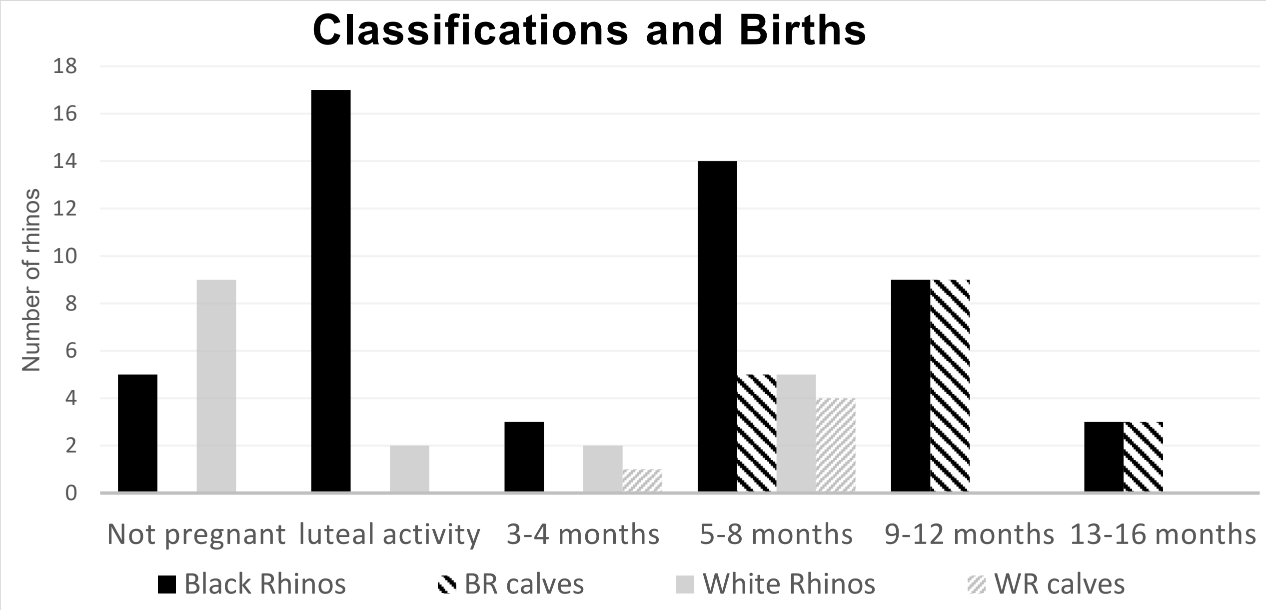
Based on their progesterone concentrations and estimated duration of pregnancy, rhinos were classified as follows:

- BR=5; WR=9 “Non-pregnant”
- BR=17; WR=2 “Luteal activity”
- BR=3; WR=2 “3-4 months pregnant”
- BR=14; WR=5 “5-8 months pregnant”
- BR=9 “9-12 months pregnant”
- BR=3 “13-16 months pregnant”

5.2 Disruption of pregnancies

None of the BR or WR classified as “luteal activity” and of the BR classified as “3-4-months pregnant” gave birth. Only seventeen BR, classified as “5-8-months pregnant” or higher (65 % of these classifications), gave birth. Post-mortem examination in one BR from this classification indicated a recent abortion. Only one WR classified as “3-4-months pregnant” (50 %) gave birth and four WR classified as “5-8-months pregnant” (80 %) gave birth. All rhinos classified as 9-12 months or higher gave birth (Figure 8).

Figure 8: The number of translocated black and white rhinos, pregnancy classifications and resulting births



As shown in Figure 9, 50 % (one out of two) of the WR classified as “3-4 months pregnant” gave birth, whereas 0 % of the BR did.

From 14 BR, classified as “5-8-months pregnant”, only four gave birth, resulting in a 36 % birth rate of within this classification. However, four out of five WR classified as “5-8-months pregnant” gave birth, resulting in a successful birth rate of 80 %.

Both classifications of “9-12-months pregnant” and “13-16-months pregnant” resulted in a 100 % successful pregnancy rate with nine out of nine BR and three out of three BR giving birth.

Figure 9: Percentage of successful births per pregnancy classifications in translocated black and white rhinos

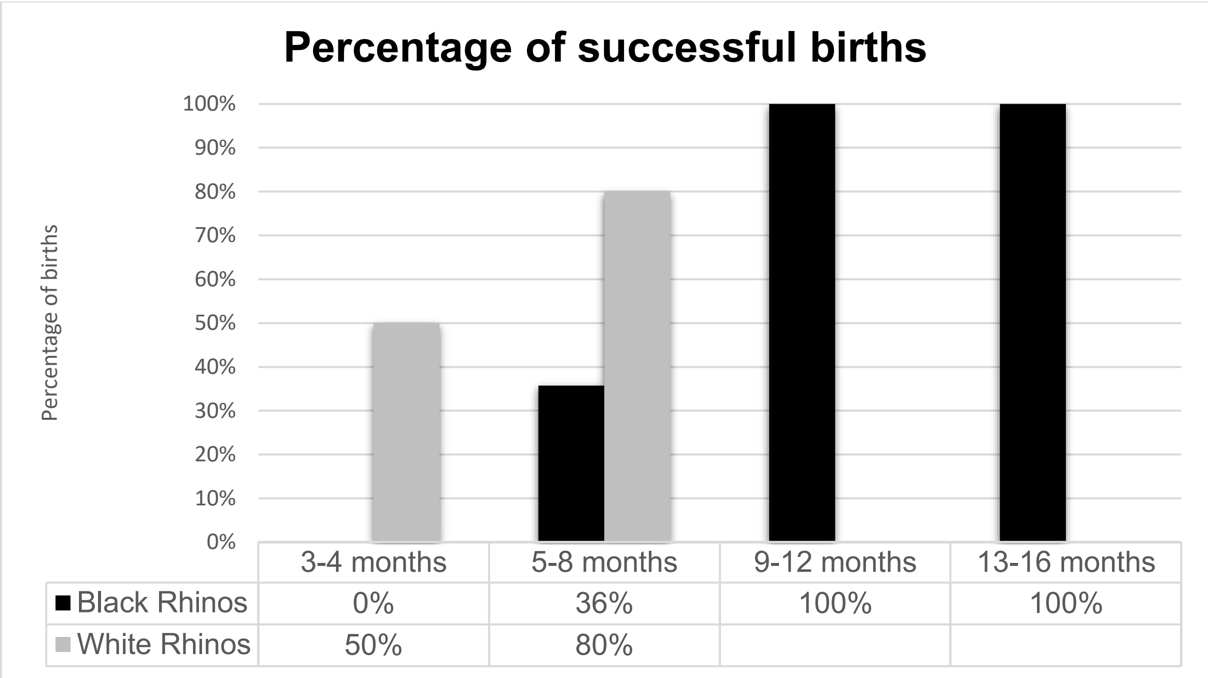


Figure 10 and Figure 11 show the percentage of possible disrupted pregnancies in black- and white rhino, respectively. Overall, BR lost 59 % of their calves, whereas WR lost 44 % of their calves.

Figure 10: Percentage of possible disrupted pregnancies in translocated black rhinos

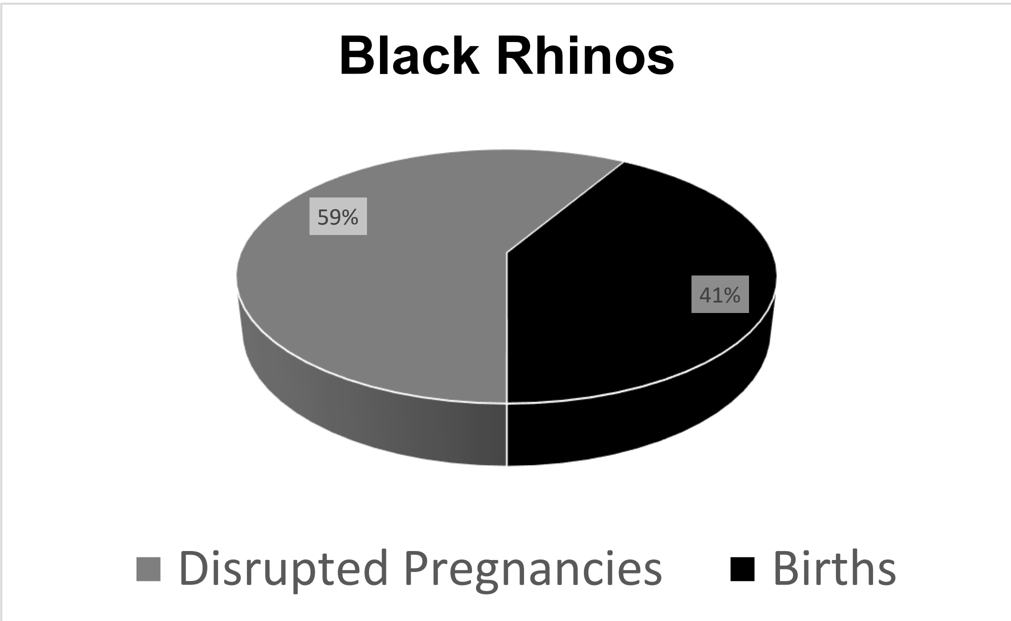
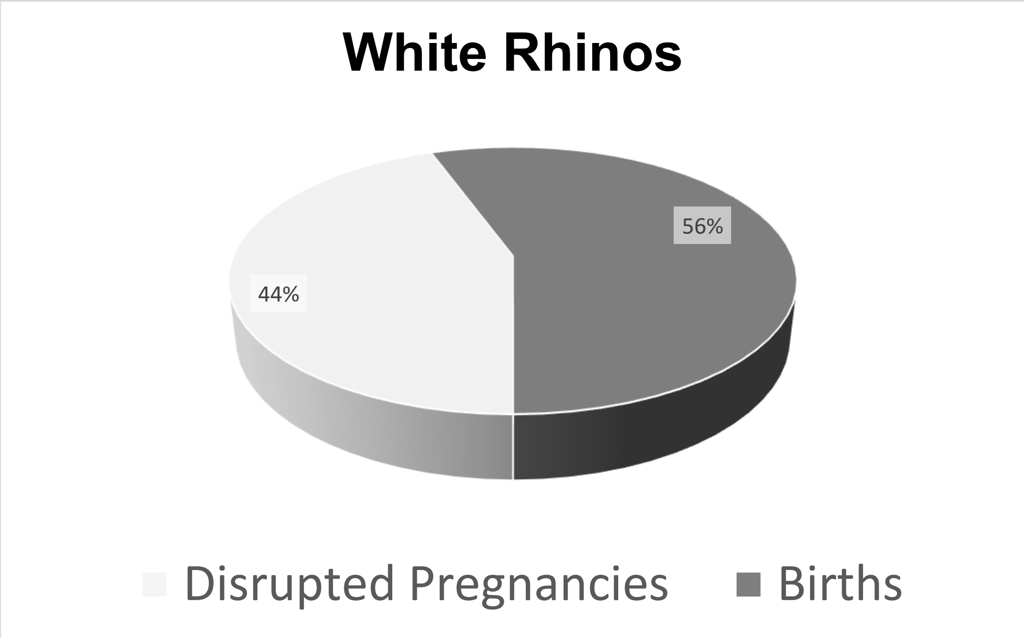


Figure 11: Percentage of possible disrupted pregnancies in translocated white rhinos



As shown in Figure 12 and odds ratio of 34.52 with 95 % CI of [1.8 - 650.6] has been calculated, which means, that rhinos in late term pregnancy (9-16 months) are more likely to give birth than rhinos in the early- to mid-term pregnancy group (3-8 months). With a P-Value of 0.0181 the association between pregnancy classifications and outcomes (births) is considered to be statistically significant.

Figure 12: Odds ratio calculator using MedCalc (www.medcalc.org) showing the results of the Odds ratio of successful births compared between early- to mid-term pregnancies and late term pregnancies, 95 % CI, z Statistic and P-Value

Results

Odds ratio	34.5238
95 % CI:	1.8321 to 650.5771
z statistic	2.364
Significance level	P = 0.0181

5.3 Sex-Ratio Bias

Seventeen BR, classified as "5-8-months pregnant" or higher, gave birth to ten male (59 %), five female (29 %) and two calves of unknown sex respectively. In detail, the BR classified as

“5-8 months pregnant” gave birth to 2 male and 3 female calves, “9-12 months pregnant” to 6 male and 2 female calves, and “13-16 months pregnant” to 2 male calves (Figure 13).

Only one WR classified as “3-4-months pregnant” gave birth to a female calf and four WR classified as “5-8-months pregnant” gave birth to three male and one calf of unknown sex. Figure 14 shows the sex ratio of successful births compared to the number of possible disrupted pregnancies. While 24 BR experienced possible disrupted pregnancies and gave birth to 10 male, 5 female and one calf of unknown sex, only 4 WR experienced possible disrupted pregnancies and gave birth to 3 male, 1 female and one calf of unknown sex.

Figure 13: Sex distribution of black and white rhino calves from different pregnancy classifications of translocated black and white rhino mothers

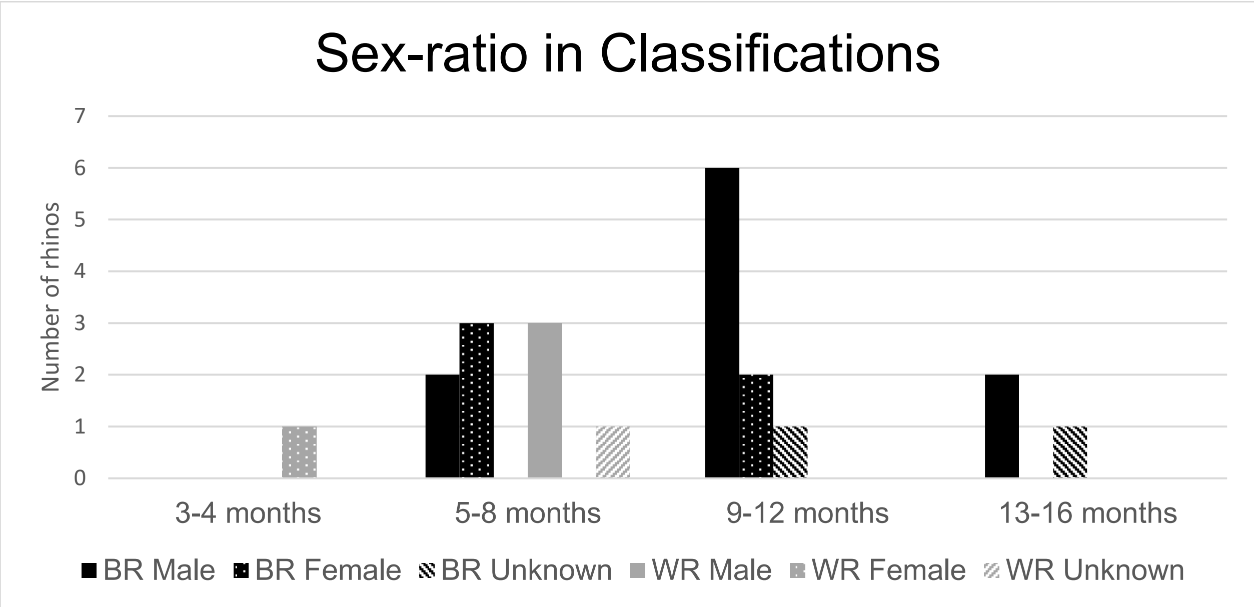
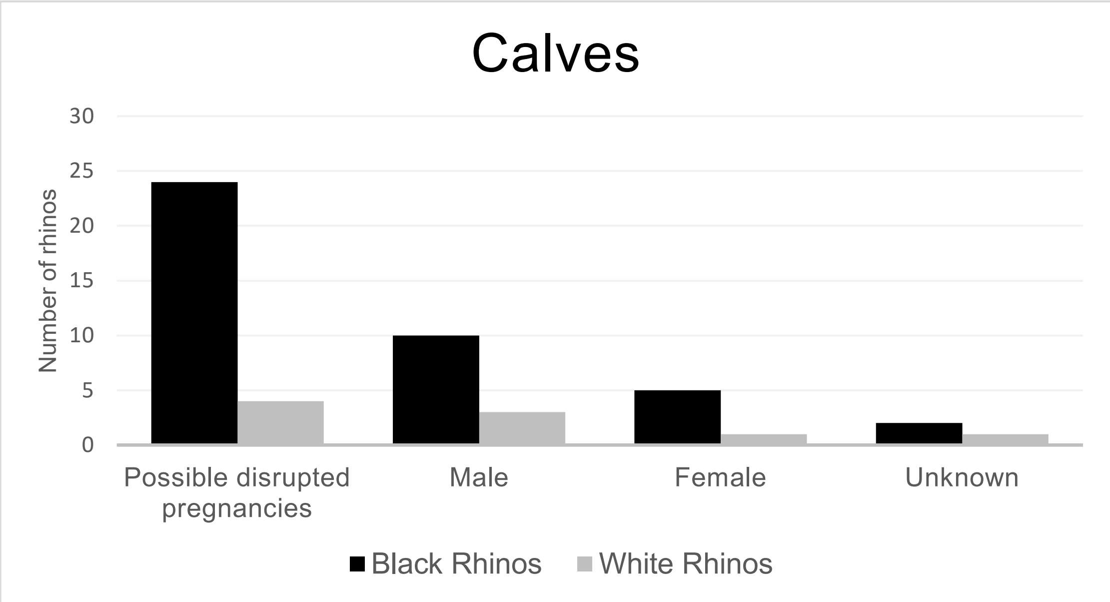


Figure 14: Black and white rhino calf sex ratios and possible disrupted pregnancies in translocated black and white rhinos.



As displayed in Figure 15 and Figure 16 the sex-ratio appeared to be skewed towards male calves in both black and white rhinos. In BR male calves were the most numerous with a percentage of 59 %, followed by female calves with 29 % and calves of unknown sex being the lowest percentage at 12 %. In WR there were similar results, with male calves forming a total of 60 %, female calves and calves of unknown sex were both at 20 %.

Figure 15: Birth-sex-ratio in black rhino calves born after translocation

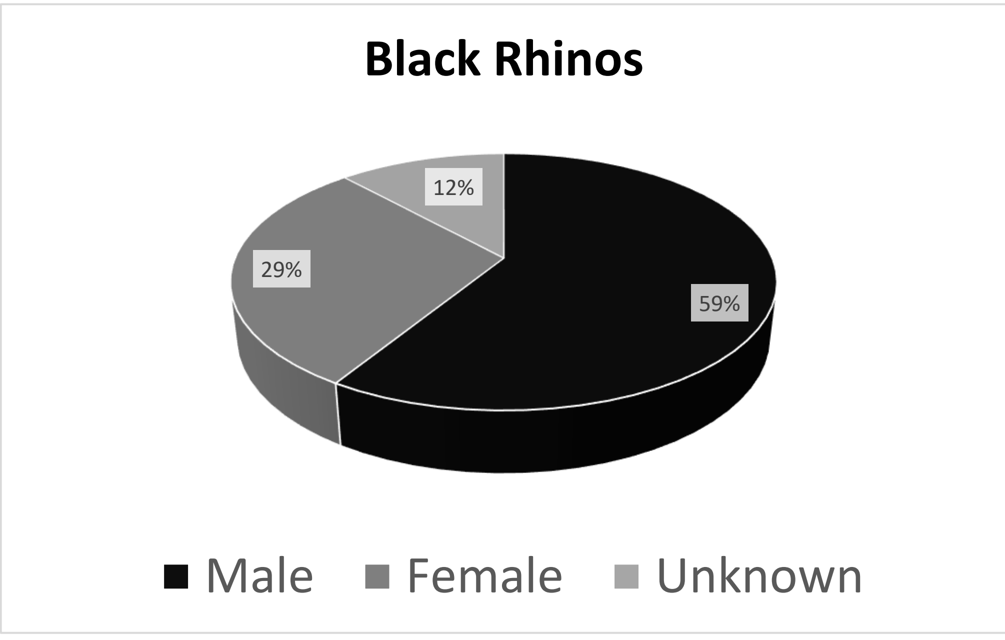
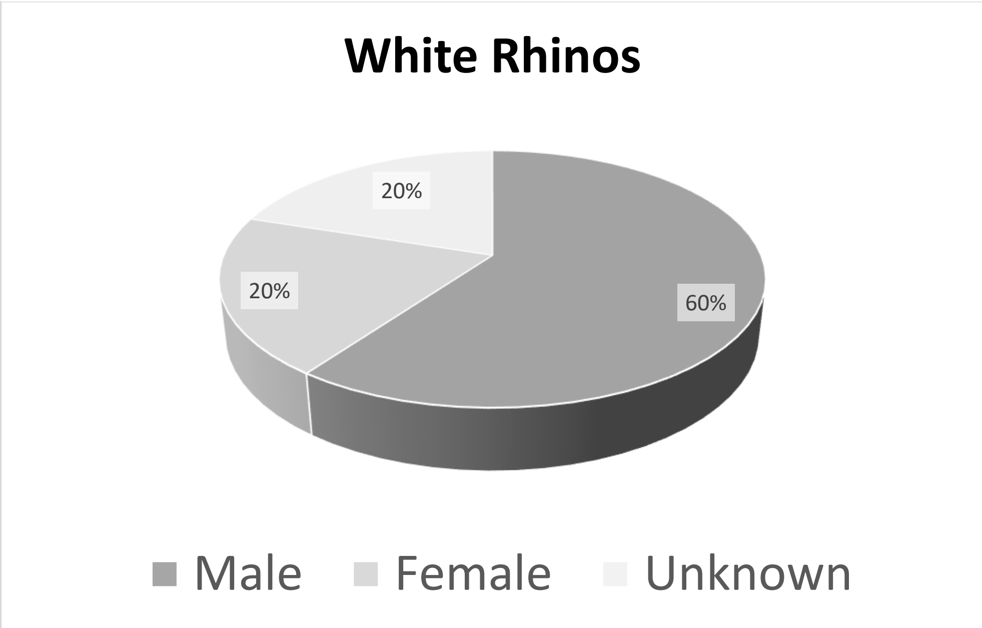


Figure 16: Birth-sex-ratio in white rhino calves born after translocation



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The odds ratio in Figure 17 of 2.2 with 95 % CI of [0.6 - 8.0] has been calculated. The CI is below and above 1, which shows that there is no difference in BSR between the transported rhinos of this study and non-transported rhinos of the founder population in the Hluhluwe–iMfolozi Park KwaZulu-Natal. The P-Value of 0.25 confirms that there are no statistically significant differences.

Figure 17: Odds ratio calculator using MedCalc (www.medcalc.org) showing the results of the Odds ratio of the sex of the calves compared between transported rhinos and non-transported rhinos from the founder population, 95 % CI, z Statistic and P-Value

Results

Odds ratio	2.1667
95 % CI:	0.5873 to 7.9933
z statistic	1.161
Significance level	P = 0.2457

When calculation odds ratios comparing male vs. female calves in 3-8 months vs 9-16 months pregnant rhinos, we found no significant differences. The odds ratio in Figure 18 of 3.2 with 95 % CI of [0.4 - 24.4] has been calculated. The CI is below and above 1, which shows that there is no difference between the two pregnancy groups. The P-Value of 0.26 confirms that there are no statistically significant differences.

Figure 18: Odds ratio calculator using MedCalc (www.medcalc.org) showing the results of the Odds ratio of the sex of the calves compared between early- to mid-term pregnancies and late term pregnancies, 95 % CI, z Statistic and P-Value

Results

Odds ratio	3.2000
95 % CI:	0.4194 to 24.4180
z statistic	1.122
Significance level	P = 0.2619

6 Discussion

This study put forth results on possible consequences of translocation stress in pregnant black and white rhinos and creates a foundation for future research. The results show that translocation stress might disrupt early- to mid- term pregnancies in rhinos, with later pregnancies having a higher chance of survival, and, although not statistically significant, there appeared to be a possible BSR towards male calves. Both observations could be related to the cortico-adrenal stress response and underlying causes require further investigation.

6.1 Underlying causes

6.1.1 Disruption of pregnancies

In general, it shouldn't come as a surprise that translocation, a high stress event, influences the hormonal balance of the respective animal. Translocation includes repeated and prolonged stress-inducing events including chemical immobilization, capture, crating, transport, handling, release and acclimation to a new environment (9,11). Consequent activation of the hypothalamic–pituitary–adrenal (HPA) axis and a glucocorticoid response induces physiological challenges in the rhino mother that may only return to pre-capture levels after the rhinos, for example, has acclimated behaviourally, socially, and physiologically to its new home (11,38).

These physiological challenges include:

1) Life-threatening respiratory and lactic acidemia as well as hypoxemia associated with the unique challenges of rhino capture (49);
2) The mounting of a stress response to capture and the novelty of transport;
3) Stress-induced immunomodulation, OS and APR; and
4) Skeletal muscle fatigue, energy imbalance and dehydration that likely become more relevant with increasing transport time (13,49).

Thus, all five domains of animal welfare, which are: 1. Nutrition, 2. Physical Environment, 3. Health, 4. Behavioural Interactions and 5. Mental State are all temporarily jeopardized in translocations, which is a trade-off for the much greater objective to save the species (50). It must be noted that these domains are interconnected and influence each other (51). For example, compromise in the first four domains (e.g., thirst-hunger, muscle injury, disease, and behavioural restriction) is expressed in the fifth domain (stress), which is also primarily affected by translocation (50).

The stress response is known to have dramatic consequences on rhino reproduction (38). The hormonal balance is incredibly sensitive, and even more so for an embryo or fetus. Any influencing factors capable of disrupting it can lead to numerous pre-partal complications, like resorption of the fetus, abortion, fetal malformations etc, (31).

Stress has reported to:

1) Induce a testosterone surge in the mother (52,53);
2) Cause extreme increases in serum glucose concentrations in the mother (54);
3) Suppress progesterone production and progesterone sensitivity in the mother (55); and
4) Cause resource deprivation for the embryo where the mother’s nutritional needs supersede those of her developing fetus (56)

Progesterone is vital for implantation and to maintaining the pregnancy (20,57). In horses, progesterone from ovarian sources is required for early pregnancy maintenance until the feto-placental unit begins taking over. Thus, if a mare was ovariectomized during early pregnancy, it would abort (58). Endogenous prostaglandin release during early gestation caused by endotoxin release has been shown to cause luteolysis and a decrease in progesterone concentrations resulting pregnancy loss in horses (58). These pregnancy losses could be mitigated by administering the synthetic progesterone “altrenogest”. Similar results were obtained by the administration of the non-steroidal anti-inflammatory Flunixin meglumine, which was useful only when administered before the endotoxin challenge (58). Interestingly, there is evidence that stress, particularly mental stress, increases prostaglandin concentrations in the body and brain (59,60).

Suppressed fecal progesterone metabolite concentrations have been reported in black and white rhinos after translocation (61). Whether serum prostaglandin concentrations increase in translocated rhinos is unclear but could be subject of future investigations. Importantly, similar to horses, results from these studies could be used to initiate hormonal or anti-inflammatory therapy in pregnant rhinos subjected to translocation aiming at mitigating possible pregnancy losses. Two studies in zoo animals described, that female rhinos with a history of pregnancy loss were supplemented with a synthetic progestin and proceeded to carry their pregnancies to term (15,17). These, and other preventive treatments, could be explored for translocated rhinos as well.

Pregnancy losses associated with rhino translocation have not been previously reported. However, there is indirect evidence that these have occurred. Oestrus and ovulation after abortion are delayed in horses (62). In rhinos, a delay in first post-release reproduction, as well as failure of females to calve, have been reported, which could indicate that pregnancy losses might have occurred during translocation (32,63). In the BR translocations reported from Kenya by Brett (1998) (28), the method for pregnancy diagnosis was the presence of a swollen udder, which is solely an indicator for a late term pregnancy and thus, early and mid-term pregnancies were likely missed and therefore not reported (32). Future research should apply validated and more sensitive tools to diagnose pregnancies and investigate possible pregnancy losses in translocated rhinos.

Moreover, there is a need to evaluate other possible causes of pregnancy loss than stress in translocated rhinos. Negative side effects of the etorphine-based rhino immobilization on fetal viability should be investigated such as acidosis, hypoxemia, hypotension, hypovolemia, and changes in body temperature (13). Also, the question if longer transport time, which equals longer sedation time has a higher risk of disrupting pregnancies in rhinos, just like it does in horses (25,30), has to be answered.

Lastly, the time after translocation and release is a crucial and stressful event for the rhino and could influence pregnancy outcomes and should therefore be closer investigated. The environment is unfamiliar to the introduced individuals, they might have difficulties in finding new water sources or good food resources, and they need to learn their way around the new area. Their behavioural and mental state are highly influenced during this time and the translocated individuals have no choice but to establish their rank in the new population, which is why fights are more frequent and could influence calving rates. It is known that fighting is a major cause of death in males, but not only male rhinos have been observed to fight (6). Females have been observed to fight similarly by, for example, actively driving off other animals (64).

6.1.2 Sex-ratio bias

Although not statistically significant, the greater odds in translocated rhinos to give birth to a male calf clearly deviates from the founder populations of the black rhinos from this study where there were no significant differences in the sex ratio of BR calves (40).

If the TWH was supported in our study, it would raise the question, why not more female calves were born. According to the TWH, as maternal condition declines, adult females tend to produce a lower ratio of males to females. Only mothers in good condition would be expected to have sufficient resources to produce surviving sons (34). It appears that male offspring are heavier at birth, born later, and suckle more frequently and until a later age than female offspring and thus, require more maternal investment (33).

Facing several stressors during translocation, research has shown that animal welfare is challenged during translocation, which likely impacts maternal condition (50). Thus, a BSR towards female calves would have been expected in our rhinos, but the opposite was observed. If the TWH was true, it could be that only the strong mothers in good condition gave birth to (male) calves, whereas weaker mothers aborted.

It can be theorized that stronger rhinos in better conditions are selected for translocations. Consequently, the rhino cow may have been in good condition on capture and if she was in the mid- to end-term stage of pregnancy, the sex of the calf would no longer change. Hence, even if the female loses condition after release, she will still give birth to a male calf.

Alternatively, it could be that the TWH, similar to the American bison, simply does not apply to rhinos. However, further studies with larger sample-sizes are required to confirm our results and closer investigate these assumptions.

According to the maternal condition hypothesis, rhino age might have also played an important role in the BSR. Very young and aging females have a lower maternal condition compared to prime breeding adults so that the sex ratio of male to female calves would be expected to be low in these age groups (33). Unfortunately, we did not have these data available for our study, which should be included in future reporting and analysis.

Another suggested hypothesis to explain skewing of sex ratios is based on the timing of fertilization with males being produced when insemination occurs close to the time of ovulation (33). This hypothesis is difficult to investigate in free ranging wild animals, but future studies in captive rhinos subjected to translocation could include these factors in their study design.

A skewed BSR in rhinos after translocation has already been documented. Morrow et al. 2019 report a sex ratio of 1:0.71 male: female in WR translocated from the Kruger National Park, SA, to New Zealand (65), whereas Linklater et al. (2007) report a BSR bias towards male calves in rhinos translocated in early gestation and towards female calves in rhinos translocated during mid-gestation (38). These findings deviate from ours, where rhinos in early pregnancy experienced possible abortion and calves born from rhinos in mid- and late pregnancy, although not statistically significant, clearly show a BSR towards male calves.

The authors proposed the following mechanisms related to a stress response to translocation that could cause sex-differential early embryo death and lead to the observed skewed BSR:

1) Sex-differential embryo death from excess glucose metabolism, associated with hyperglycemia and stress causing female embryo mortality. This effect is greatest during the implantation phase of pregnancy (38). As none of the rhinos from our study that were this phase of pregnancy gave birth to a calf, we believe that this mechanism is a rather unlikely cause for the observed BSR in our rhinos but could have contributed to the possible pregnancy losses.
2) Asynchrony between uterine and embryo development. Stress and hyperglycemia could interfere with or exacerbate a developing asynchrony between the embryo and uterus by delaying a progesterone surge or the readiness of the uterus (38). Rhinos are known to experience a stress response and associated hyperglycemia to capture and transport (12). Thus, this proposed mechanism could have contributed to the observed BSR in our animals.
3) Pregnancy hormone suppression and resource deprivation: Stress may modify the relationship between parental energy balance, or the ability of investing into sons or daughters (costs vs. benefits). The RWB rhinos are known to have entered a negative

energy state during translocation, which might have contributed to this mechanism of BSR (13).

4) It has been suggested that no more sex-bias occurs in late-gestation in rhinos (last 1/5th) (38). In this study all rhinos staged over 9 months pregnant gave birth, which acts in accordance with this suggestion. However, the number of male calves (n=8) is much higher than that of females (n=2) in this group.

Future studies measuring the effects of these multiple mechanisms and modelling their combined outcomes are required to improve our understanding of what drives BSRs in translocated rhinos.

6.2 Limitations of this study

The first most pressing limitation of this initial study is that translocations took place independently. Consequently, it was impossible to create a controlled environment and standardized conditions. However, we believe that therefore our results represent a “real-life” setting where not everything is always perfect and thus, show high ecological validity (40).

This study also uses historical data, where we have very little knowledge of the given circumstances like the anesthetic medication administered, different handling of the rhinos, varying durations and techniques of anesthesia and translocation, and even unique challenges in their new environment and much more.

Moreover, the assumption is that all calves born post-translocation were spotted by the monitors. Due to the high juvenile mortality rates, and given that the cows were in new environments (this is especially true for cows in late-term pregnancy that would not have had much time to settle down before giving birth) it is possible that calves were born to cows indicated as pregnant but were lost to predation, abandonment or other causes (e.g. stuck in mud) before the monitors picked up the calf. Therefore, the post-translocation birth success could potentially be higher than we assume because calves died before their presence was noticed.

In some rhinos there are no recordings about the actual quality of blood samples and how they were collected. Proper handling of the samples affects the reliability of the interpretation of results. For example, wrongly using gel tubes leads to falsely low progesterone concentrations, as progesterone binds to the gel, or hemolysis as a result of not separating the serum within the proper timeframe might also result in falsely low progesterone concentrations as metabolism by blood cells occurs (66). These variables could have possibly influenced the results.

The second important limitation is that this study relies heavily on the yet to be validated serum progesterone concentrations and estimated duration of pregnancy. Hence, there is evidence that there are interfering factors disrupting the pregnancies and the BSR in translocated rhinos, but it is difficult to confirm this assumption until these tests are validated. The question of how accurately the pregnancy classifications were determined by serum progesterone concentrations still needs to be answered but is subject of a current validation study by Prof. Henk Bertschinger. However, at its current stage, the validation process is met by various problems, including lack of reporting and long-term monitoring of rhino births by stakeholders and veterinarians, who have submitted samples. The information provided in this thesis represents the newest and most accurate state of knowledge based on the available data that is currently being used to validate the serum progesterone concentration-based rhino pregnancy classifications.

Another important limitation of this study is the fact that in nine BR, estimated months of pregnancy at transport were only back-calculated based on the available calving records and no blood samples were taken. These data are useful when determining BSR and birth success of these late-term pregnant rhinos, however, other rhinos from the same cohort could have been pregnant and could have aborted without anyone noticing.

Also, the comparison from the five individuals, where we have both, the progesterone concentrations and the pregnancy estimates show, that in these five animals the pregnancy was determined with a 100 % sensitivity. But the accuracy of the months was only correct in 40 % (n=2), with a small deviation from 1-2 months in 40 % (n=2) and a deviation from three months or higher was shown in 20 % (n=1). Moreover, there might be inter-species differences between black and white rhinos, that we did not consider in this study due to the relatively small sample size.

Regarding the calculation of OR, with an Odds ratio this high it is important to state that if the odds ratio is interpreted as a relative risk, it will always overstate any effect size: the odds ratio is smaller than the relative risk for odds ratios of less than one, and bigger than the relative risk for odds ratios of greater than one (67).

Despite these limitations, we believe that this study provides valuable information that helps guiding future research. This study is meant to be an initial step towards further investigations that, after careful validation of methods, are needed to confirm these results and obtain more complete datasets.

7 Conclusion

Due to poaching, black and white rhinos are endangered or threatened species at the present time. Translocations are crucial for conservation and have shown to be a very successful strategy to manage viable populations. Since the poaching pressure doesn't offer great prospects and doesn't seem to cease, it is more important than ever to improve rhino translocation strategies and enhance the chances of post-release survival of calves and reproductive efficacy.

7.1 Future Perspective and Possible Solutions

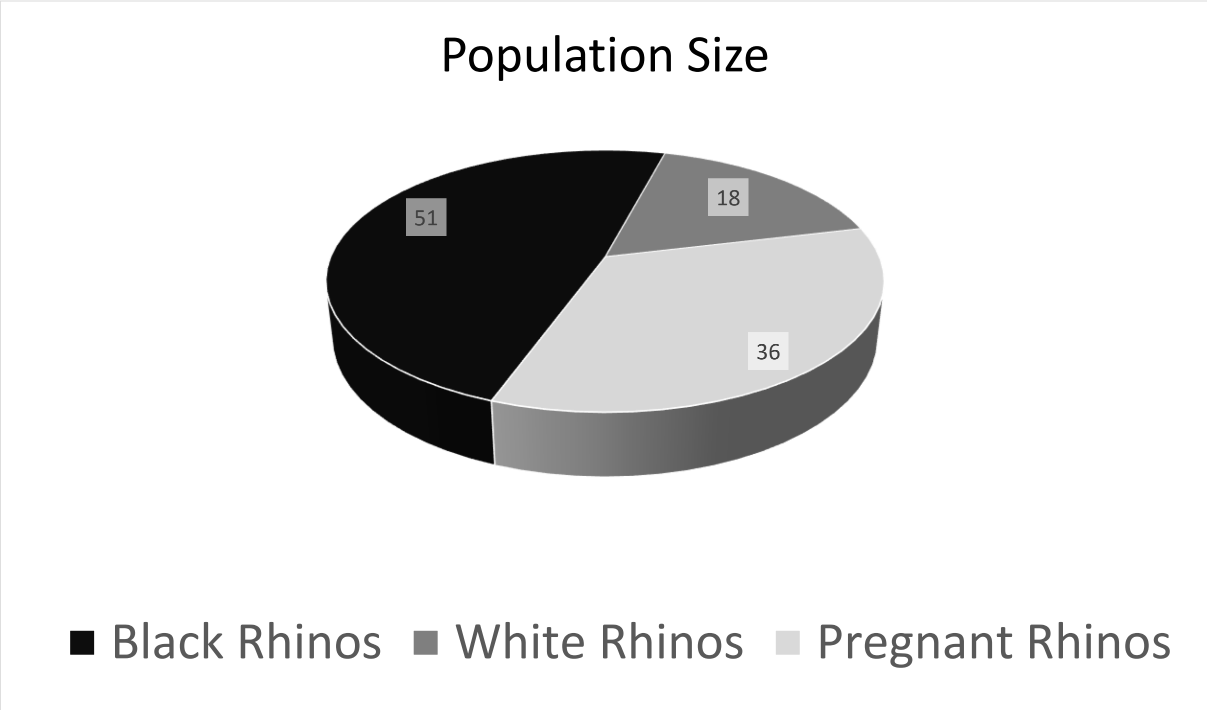
Possible management solutions and modifications are important in order to react without further ado to the results of this initial study with the conservation aspect in mind.

The first proposed approach is one simple and controversial approach for future studies. Its purpose is to keep in mind how many animals are truly lost with each translocation. The method is to count pregnant rhinos as two individuals.

Here is a thought experiment on how we could have counted the possible pregnancy losses as general losses in our study. This serves to make disrupted pregnancies more visible.

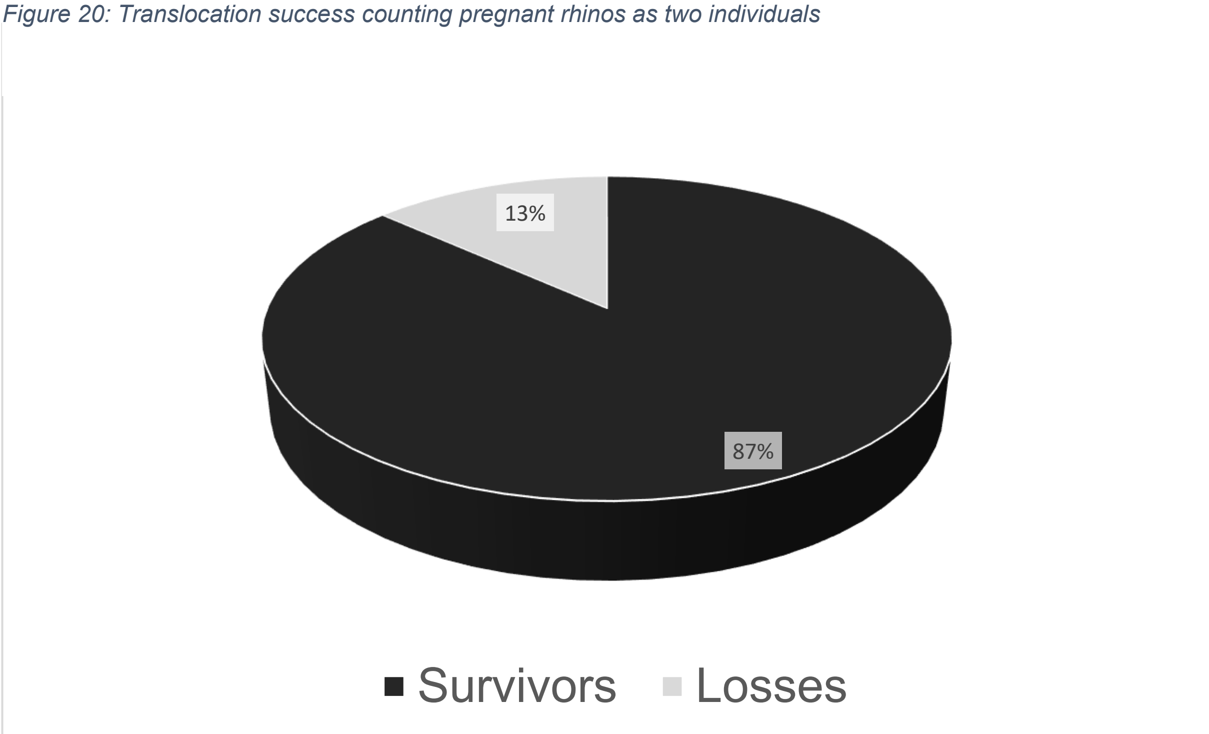
If we had counted the pregnant rhinos as two individuals instead of 51 black rhinos plus 18 white rhinos, equals 69 rhinos we would have to add 36 pregnant rhinos, again excluding the ones classified as "luteal activity" as seen in Figure 19.

Figure 19: Population size of the current study counting pregnant rhinos as two individuals



We would then have a total of 105 rhinos out of whom only 91 survived, which is a mere 87 %, as seen in Figure 20. Which means in such an undergoing we are losing 13 % of our rhinos which we are trying to protect. These are solely the calves that might have been born, if their mothers hadn't been transported while pregnant, based on the assumption that monitors detected all calves that were born. The 13 % don't include any other losses post translocation such as intra- or interspecies conflict (with other rhinos, elephants etc.), predation, poaching, etc. If you compare these embryo losses to regular losses, which are about 1-8 % in different studies, the translocation failure rate for rhinos is comparatively high (64,68).

Figure 20: Translocation success counting pregnant rhinos as two individuals



Another possible solution would be to investigate more accurately prior to the translocation if the rhino is pregnant. This is already possible in captive rhinos with non-invasive pregnancy tests through urine and feces (69). It is already an established and reliable method for farm and zoo animals and can also be used in wild animals (16,70).

Further validation of pregnancies with additional methods, like ultrasound, blood relaxin concentrations, vaginal cytology, and rectal palpations with short durations of anesthesia would also be a possible solution. Ultrasonography represents an easy method to receive additional information, like a proper reproductive assessment including the reproductive status and a ruling out of pathologies of the whole reproductive tract.

Relaxin, a pregnancy specific hormone, produced by the fetoplacental unit, has already successfully been used to diagnose pregnancies in Sumatran rhinos. Homologous porcine relaxin was used in these efforts. The concentration surges in between 7 to 10 months of pregnancy and showed promising effects do detect later pregnancies in rhinos. As there is no white or black rhino specific relaxin assay, this means assay kits from different species have to be evaluated to see if any of them are suitable (22,27).

Translocations are an important biological management tool and there would not be the current number of rhinos in the world if there were not risky translocations undertaken. So, the translocation methods need to be under continuous improvement and revised regularly (3). This initial study is supposed to inform about possible implications of translocation on rhino biology and translocation management.

The ultimate goal of this thesis was to help improve the outcome of rhino translocation by understanding the effects of translocation stress and informing possible translocation protocol and management adaptations to contribute towards conservation of the species. The information gained from this thesis, importantly, has paved the way for further studies investigating pregnancies and possible pregnancy losses in translocated rhinos with more sophisticated methods and under more controlled settings. Should these studies confirm these initial results, the question might be asked whether it is appropriate to translocate female rhinos in an early- or mid- term stage of pregnancy or if there are any interventions that can be applied to mitigate these losses and improve the success of rhino conservation translocations.

8 List of Tables

9 List of Figures

10 References

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