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Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation

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Abstract

White rhinoceroses suffer from a low rate of reproduction in captivity. This study examines the role of male reproductive function as a contributing factor. We used ultrasonography to image accessory sex glands, testis and epididymis. Electroejaculation provided 36 ejaculates from 21 rhinoceroses. Based on the percentage of progressively motile spermatozoa, semen was categorized in three groups, high (I: >75%), intermediate (II: 50–70%) or low (III: <50%) quality. Only 52% of the males showed high semen quality. Ejaculates in the high motility category also had the highest proportion of morphologically intact spermatozoa. Both semen parameters, sperm motility and morphology, were found to positively correlate with size of the accessory sex glands. The semen category was associated with group size suggesting that the social status influenced functional reproductive parameters. Change of territorial status ($n = 1$) improved semen quality. Testicular fibrosis was characterized as a sign of reproductive ageing in all males older than 15 years of age ($n = 13$); although, this ageing process did not notably affect semen parameters. Furthermore, for the benefit of assisted-reproduction and genetic banking programs protocols for the storage of cooled semen and the cryopreservation of spermatozoa were designed using different cryodiluents. This report provides basic data for the evaluation of reproductive components and of breeding management in male

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rhinoceros. Our results indicate that low rate of reproduction in captivity can be attributed to reduced male reproductive fitness. Changes in management of white rhinoceroses may positively affect male reproductive function.

Keywords: White rhinoceros; Reproduction; Genital ultrasound; Ejaculate; Cryopreservation

1. Introduction

Substantial knowledge on the reproductive physiology of the female white rhinoceroses has been gathered over the last 10 years [1,2]. The impetus for these studies was a low rate of reproduction in the captive population [3]. Acyclic and erratic luteal activity, mating, conception and pregnancy failures and reproductive ageing have been identified in female white rhinoceroses that have low rate of reproduction [1,4–6]. These problems are currently being addressed [7]. However, little emphasis has been put on the evaluation of male fertility. Considering that 81% of all male white rhinoceroses in the Species Survival Programs have not yet sired offspring [8], male fertility may represent a contributing factor to the reproductive failure of the captive population. Besides the description of different semen collection methods and basic reproductive data for three rhinoceros species [9,10], to date analysis of reproductive fitness in a significant number of non-reproducing males has not been performed. Clinical examination of reproductive organs and assessment of semen quality have historically been used to predict potential fertilizing capability of stallions [11,12].

In this study, reproductive tract ultrasonography and electroejaculation were used in tandem to obtain information on the reproductive parameters of the male white rhinoceros. The purpose of this study was to compare the reproductive efficacy of mature, non-reproducing males managed in groups with different male to female ratios. The effects of changing the housing management of male and resulting upgrade in social status on reproductive factors were instigated in one case. For the prevention of extinction and the improvement of assisted-reproduction programs and sperm banks of endangered species [13], freshly collected semen via electroejaculation was used to design protocols for storage of cooled semen and cryopreservation. This is an essential prerequisite for artificial insemination in rhinoceros.

2. Materials and methods

2.1. Histology

Prior to the main investigations of this study, the genital tracts of two male rhinoceroses (28/21 years) were examined post mortem to establish further baseline values to existing data [10,14]. The genital tracts were exenterated, frozen at −20 °C during shipment and dissected after thawing. The morphologic and ultrasonographic characteristics of the genital tracts were ascertained in a water bath before the testis, and epididymis were fixed

in 10% formalin. After fixation, tissue samples were embedded in paraffin blocks. Tissue sections (4 mm) were cut and stained by routine methods with haematoxylin eosin (HE) and Azan for histological examination.

2.2. Animals

Male reproductive status was assessed in 19 mature, clinically healthy Southern white rhinoceroses (*Cerothoterium simum simum*) and two Northern white rhinoceroses (*Ceratheterium simum cottoni*) (Table 1). All animals but one were listed in the European and North American Species Survival Programs (SSP), which are dedicated to the propagation of this species in captivity providing the best possible husbandry. Males were housed solitarily ($n = 4$), as single male with one to three females ($n = 7$), as two males with three to five females ($n = 3$), or as multi-males with five males and six females ($n = 1$). Four males of the study group were proven breeders, No. 245, 487, 497, and 974. However, last successful breeding of these males had been 8, 5, 12 and 3 years, respectively prior to this reproductive assessment. Mating had been observed in only three males (No. 487, 532, 974). All except the solitary housed males ($n = 5$) and the male, No. 974, had access to one or more reproductively healthy females (Hermes, unpublished). The management of the multi-male group, from which the social structure had been analysed and reported [15], was changed after the initial reproductive assessment of all males. The large enclosure housing all males was divided into two separate enclosures. One enclosure housed a formerly subordinate rhinoceroses (No. 497) with exclusive access to cycling females. All other males including the dominant male were housed in a second enclosure without physical access to the cycling females but with visual and tactile contact. Males were not mixed between enclosures at any time. Male No. 497 was re-examined six months after management changes had been implemented.

2.3. Anaesthesia

Semen collection using electroejaculation required general anaesthesia. All animals were anaesthetized using etorphine at a per-animal mean (±S.E.M.) dose of 3.1 ± 0.6 mg; acepromazine at a dose of 12.5 ± 2.5 mg (Large Animal Immobilon® C-Vet Veterinary Products, Lancs UK); detomidine-HCl at a dose of 12 ± 2 mg (Domosedan®, Orion Corporation, Farmos Finland); and butorphanol at a dose of 12 mg (Turbugesic®, Fort Dodge Animal Health, IA). Anaesthetics were injected into the neck muscles caudoventral to the ear using a dart pistol and 3.5 ml plastic darts with a 60-mm needle (Daninject International Gelsenkirchen, Germany). An additional IV injection of ketamine at a dose of 150 ± 100 mg (Narketan®, Chassot AG, Bern, Switzerland) injected into the ear vein was used to reduce the time to lateral recumbence. In several cases, xylazine at a dose of 40 ± 20 mg (Rompun®, Bayer AG, Leverkusen, Germany) was administered to facilitate better muscle relaxation. A heavy-duty inner tube was placed beneath the shoulder and pelvis in order to alleviate possible compressive trauma to the downed side during lateral recumbence. All animals received supplemental oxygen at a rate of 15 L/min through a nasal tube. Anaesthesia was reversed in all cases with an IV combination of 250 mg of naltrexone (Trexonil®, Wildlife Laboratories Inc., Fort Collins, CO) and 20 mg

Table 1
Semen collections in 21 white rhinoceroses (*Cerathoterium simum simum, C.s.s.; Cerathoterium simum cottoni, C.s.c.*) sorted by group size

Studbook number	Generation	Name	Age (years)	Institution	Breeder	Mating	Group size	Semen collections	Season (month)	Semen category
Multi-male + females										
245, *C.s.s.*	0	Rafi	30	Zoological Center, Ramat Gan, Israel	Yes	No	5.6	1	May	II
487, *C.s.s.*	1	Shalom	23	Zoological Center, Ramat Gan, Israel	Yes	Yes	5.6	1	May	I
497, *C.s.s.*	1	Atari	22	Zoological Center, Ramat Gan, Israel	Yes	No/yes*	5.6	2	May (2×)	III/I*
913, *C.s.s.*	1?	Zion	14	Zoological Center, Ramat Gan, Israel	No	No	5.6	1	May	II
1049, *C.s.s.*	1	Zafriel	10	Zoological Center, Ramat Gan, Israel	No	No	5.6	1	May	III
Two males + females										
363, *C.s.s.*	0	Lucifer	31	African Lion Safari, Cambridge, Canada	No	No	2.4**	1	October	I
360, *C.s.s.*	0	Denny	28	Salzburg Zoo, Salzburg, Austria	No	No	2.3	4	March (2×)/June/ December	II
676, *C.s.s.*	1	Benno	18	Salzburg Zoo, Salzburg, Austria	No	No	2.3	4	February/March/ June/Decmber	I
532, *C.s.s.*	1	Dale	19	Burger's Zoo, Arnhem, Netherlands	No	Yes	2.5	3	March/October	I
1048, *C.s.s.*	1	Smoske	6	Burger's Zoo, Arnhem, Netherlands	No	No	2.5	1	January	II
Single male + female(s)										
160, *C.s.s.*	0	Patrys	30	Prague Zoo, Prague, Czech Republic	No	No	1.1	1	June	I
770, *C.s.s.*	1	Grace	17	La Palmyre Zoo, La Palmyre, France	No	No	1.1	2	October/November	I
974, *C.s.s.*	2	Harry	12	Alwetter Zoo, Münster, Germany	Yes	Yes	1.3	1	February	I
348, *C.s.c.*	0	Angalifu	29	Wild Animal Park, San Diego, USA	No	No	1.2	1	October	I

945, *C.s.s.*	2	Carni	12	Biblical Zoo, Jerusalem, Israel	No	No	1.2	1	May	II
578, *C.s.s.*	1	Easy Boy	22	Budapest Zoo, Budapest, Hungary	No	No	1.1	2	September/February	III
589, *C.s.s.*	1	Willi	20	Schwerin Zoo, Schwerin, Germany	No	No	1.1	2	September/October	III
Single male without female										
1, *C.s.s.*	0	Hlamb.	39	Zoological Garden, Berlin, Germany	No	No	1.0	4	January/March/ May/October	I
748, *C.s.s.*	2	Ugly	19	Granby Zoo, Granby, Canada	No	No	1.0	1	October	I
n.l., *C.s.s.*	?	Pablo	~20	Safari Park, Gänserndorf, Austria	No	No	1.0	1	June	I
630, *C.s.c.*	1	Suni	21	Zoo, Dvùr Králové n.L., Czech Republic	No	No	1.0	1	July	I

I: Progressively motile spermatozoa >75%; II: progressively motile spermatozoa 50–75%; III: progressively motile spermatozoa <50%. Season and semen category of the collections are indicated.

[*] Mating and high semen category was observed after this male had been housed as a singelton.

[**] The second male in this facility had intermediate semen category assessed by means manual semen collection only.

of atipamezole (Antisedan®, Orion Corporation, Farmos Finland). All animals were standing and alert approximately 2 min following administration of the antagonists [16].

2.4. Ultrasonography

Reproductive assessment consisted of an ultrasonographic examination (SonoSite 180 Plus, C60 2–4 MHz probe, Product Group International Inc., Lyons, CO 80540) of the genital organs and a microscopic evaluation of semen. Electroejaculation was used to collect the semen samples. Although the male rhinoceros can be trained to stand for reproductive ultrasound without sedation [10], in this study ultrasound was performed under general anaesthesia, as the first part of the reproductive assessment, immediately followed by the electroejaculation. To ensure acoustic coupling of the ultrasound waves, an enema was given prior to the transrectal ultrasound and dirt was cleaned from the inguinal area preceding transcutaneous ultrasound. Transrectal and transcutaneous ultrasound were implemented to image the intra-pelvic accessory sex glands, the testis and epididymis in the inguinal area. The accessory sex glands, testis and epididymis were measured as still images during the actual examination with standard calibrated software included in the ultrasound unit and more precisely, in retrospective analysis from recorded videotapes (Sony, Watchman, GV-D 900 E, Germany; Sony, DVM 60, Mini DV Cassette, Germany) using an image analysing program (analySIS PRO 2.10.100, Soft-Imaging System GmbH, Münster, Germany).

Measures of the length, the height and the width of the accessory glands were taken from transverse and cross-sectional still images. Since the paired sex glands, seminal and the bulbourethral gland, are cigar-shaped and round elongated ellipsoid organs, respectively, the approximate volume of these glands was calculated, according to the volume of a rotation ellipsoid accommodating the symmetrical shape of these organs [14,17, Hildebrandt, unpublished data]. The volume of both prostate lobules was calculated using the same equation. Even though the prostate lobules are oval in a transverse plane and triangular in a cross-sectional plane, the calculation of a rotation ellipsoid provided the closest approximation to the in vivo prostate volume of a rhinoceros. Only the maximum diameter of the testis and epididymis was measured. The testicular length, which exceeded the ultrasound range and the high inguinal position of the testis resulting from anaesthesia made accurate and consistent measurements of multiple dimensions difficult. Accurate values of testicular or epididymal volume were therefore not obtained.

2.5. Semen collection and assessment

Semen was collected by means of electroejaculation (Seager model 14, Dalzell USA Medical Systems, The Plains, VA, USA). The hand-held electrical probe was specifically designed for rhinoceros and stimulated both the accessory sex glands and the urethra. The individual position of each organ had been determined by the preceding ultrasound [18]. The electro probe (Dalzell USA Medical Systems, The Plains, VA, USA) expanded the lumen of the rectum providing maximum electric coupling of electrodes by spreading the rectal mucosa (Göritz, unpublished). Water (1–2 l) administered into the rectal ampulla shortly before the start of stimulation further enhanced electric coupling of the probe and reduced the amount of voltage (5–20 V) and amperage (200–900 mA) necessary for

ejaculation. A total of three to four sets of three to four electrical stimuli were applied with increasing voltage and amperage. Each set of stimulations was followed by manual massage of the pelvic and penile aspects of the urethra [10]. Collection bags placed over the penis funnelled the semen sample into a foam insulated 50 ml vial located at the bottom of the bag. Urine contamination of the ejaculate can be a problem if the stimulation is conducted "blindly" without a preceding ultrasound examination. Ultrasound imaging of the urinary bladder and the bladder neck avoided inadvertent stimulation of the bladder preventing urine contamination of the semen sample. However, over-stimulation of the urethra at the end of the electroejaculation might result in urination, which is signalled by strong pulsation of the penis and urine spraying. If urination occurred at end of the stimulatory process the procedure was terminated.

Semen samples were immediately diluted (1:1) with pre-warmed (37 °C), cryoextender BC (Berliner Cryomedium). Semen assessment included total volume, spermatozoal concentration, total sperm number, sperm motility and morphology. Sperm concentration was assessed with a haemocytometer (Neubauer, improved). For evaluation of sperm motility and morphology, an aliquot (10 μl) from each sample was diluted 1:20 in cell culture medium M199 (Sigma, Deisenhofen, Germany). All sperm samples were incubated for 15 min at 37 °C. After incubation the percentage of progressively-motile spermatozoa was assessed using a phase contrast microscope equipped with a warm stage (Olympus CH 40).

The BC diluent [19] is based on a buffer solution containing 2.41% (w/v) TES, 0.58% (w/v) Tris, 0.1% (w/v) fructose and 5.5% (w/v) lactose. A volume of 100 ml buffer was supplemented by 20% egg yolk (final concentration 15.6% (v/v)), 8 ml DMSO (final concentration 6.25% (v/v)) and 20 I.U. α-tocopherol/ml. The total mixture was stored at 4–6 °C overnight and subsequently separated by centrifugation (4500 g for 30 min). The supernatant containing the protective component of water-soluble, low-density lipoproteins was then used for semen preservation. BC was chosen as standard sperm diluent over commercial extenders because it has proven effective in preserving semen from a variety of endangered species including very sensitive elephant spermatozoa [19,20].

Semen categories were defined according to the percentage of progressively-motile spermatozoa as high (I: ≥75%), intermediate (II: < 75% and ≥50%), and low (III: <50%) semen quality. Smears were prepared from the cell suspension and the morphologic integrity of 200 spermatozoa was evaluated after staining with aqueous solutions of (1) Congo red (saturated) for 2 min, (2) 2% (w/v) tannic acid for 3 min and (3) 2.5% (w/v) brilliant cresyl blue for 30 s. The slides were rinsed between the staining steps and air-dried after staining. This procedure has been described for bull spermatozoa [21] and was useful for characterization of spermatozoa in several other exotic species [22] Spermatozoa were categorized as intact or abnormal cells. Abnormalities included defects of the head (micro- and macrocephalus, partially or completely detached acrosome), or detachment of the tails and heads from the mid-piece flagellum.

2.6. Semen preservation and cryopreservation

In order to determine the sensitivity of semen to chilling, ejaculates ($n = 7$) with high sperm motility (81 ± 9% S.E.M.) were diluted (BC, 1:1) in 50 ml tubes placed in the refrigerator at 4–6 °C. The 30 min post collection samples had reached room temperature

(RT) of 20–23 °C. The final temperature of 4–6 °C was reached within 90–120 min (initial cooling rates, 0.15–0.20 °C/min) depending on the ejaculate volume. After 24 h of chilling the samples were equilibrated for 15 min to RT and incubated for 10–15 min at 37 °C before motility and morphologic integrity of spermatozoa were evaluated.

Fourteen ejaculates from twelve males with sperm motility of ≥50% were cryopreserved using BC as the cryodiluent. Samples diluted 1:1 were centrifuged (800 × *g*) for 10 min at room temperature (20–23 °C) to eliminate seminal plasma from the ejaculate. After the removal of the supernatant, samples were re-extended with BC to four times the native sample volume. Samples were equilibrated for 2 h at 4 °C, frozen in 0.5 cc straws (Minitube GmbH, 84184 Tiefenbach, Germany) 2 cm over −80 °C liquid nitrogen vapour for 15 min before plunged into liquid nitrogen. Semen straws were thawed in a 38 °C water bath for 60 s and evaluated after 10–15 min in 37 °C water bath.

In a comparative trial, the ejaculates of five males were treated as described above except, they were divided into four aliquots and each donor's sample was extended with either BC or one of three commercial cryodiluters which included: Biladyl, Gent and Kenney (Minitube GmbH, 84184 Tiefenbach, Germany). The basic compositions of these extenders consisted of a standard-medium for bulls (Biladyl, supplemented with 16.5% egg yolk and 6.25% DMSO) or for stallions (''Gent'' and KM, modified Kenney-extender with 16.5% egg yolk and 6.25% DMSO).

2.7. Statistical analysis

All values are given as means ± S.E.M. The Kruskal–Wallis-test (non-parametric ANOVA) was used to examine the differences of measured semen parameters between the three groups (I–III) of ejaculates with subsequent Dunn's multiple comparisons test. The Kruskal–Wallis-test was chosen because standard deviations were significantly different between means of categories for several different parameters (Bartlett's test). Differences between proportions of motile spermatozoa before and after chilling of semen (4 °C) for 24 h were examined by the paired t-test; the normality was confirmed by Kolmogorov–Smirnov-test. The Spearman rank correlation coefficient was calculated to characterize the relationships between accessory gland volumes and ejaculate parameters. All calculations were performed using the SPSS 9.0 (SPSS Inc., Chicago, IL) statistical software package. The significance level was set to 5%.

3. Results

3.1. Semen collection and assessment

A total of 34 ejaculates were collected from 21 male rhinoceroses. The mean ejaculate volume of a white rhinoceros was 80.2 ± 13.0 ml. Semen quality varied considerably between rhinoceroses. The morphologic integrity of spermatozoa also differed significantly between the progressive-motility categories ($P < 0.01$; Table 2). The percentage of motile spermatozoa showed a significant positive correlation to the percentage of morphologically intact spermatozoa (Spearman $r = 0.576$, $P < 0.001$). The most prominent

Table 2
Semen parameters in 34 ejaculates from 21 white rhinoceroses, categorized according to the percentage of progressively motile spermatozoa

Semen category	Sperm motility (%)	Intact sperm	Volume (ml) (%)	Sperm concentration (10^6/ml)	Total sperm (10^9/ejaculate)
I ($n = 21$)	86.8 ± 1.3[a]	75.8 ± 3.2[a,b]	67.4 ± 16.4[a]	75.8 ± 15.6[a]	2.8 ± 0.8[a]
II ($n = 5$)	67.0 ± 3.4[b]	52.3 ± 7.4[c]	55.5 ± 18.0[a]	40.8 ± 18.8[a,b]	1.3 ± 0.4[a]
III ($n = 8$)	33.8 ± 6.7[b]	60.8 ± 9.1[b,c]	116.5 ± 33.4[a]	18.9 ± 9.9[b]	1.1 ± 0.3[a]

The columns with different superscripts (a, b) are significantly different (Dunn's multiple comparisons test).

Table 3
Semen characteristics of one white rhinoceros (Benno, No. 676) collected in four different months

Month	Volume (ml)	Sperm concentration (10^6/ml)	Total sperm (10^9/ejaculate)	Sperm motility (%)	Sperm intact (%)	Semen category
February	28.0	85.4	2.390	85	90	I
March	21.5	7.7	0.165	90	97	I
June	17.5	67.4	1.180	85	81	I
December	204.0	3.9	0.790	85	59	I

morphological abnormalities were partially or completely detached acrosomes (7–20%) or separated heads and tails (1–29%). The proportion of spermatozoa with protoplasmic droplets was below 4% in all samples. The values of the ejaculate volume, sperm concentration or total number of sperm per ejaculate did not show significant differences between the three groups.

Six of the 21 males were available for two to four collections during this 4-year study each showing consistent ejaculatory results. High as well as reduced semen qualities of all males occurred equally throughout the year (Table 1). In one male collected in four different months, semen quality was consistently high (category I) and other parameters varied without a seasonal pattern (Table 3).

3.2. Ultrasound

Prior to semen collection, the dimensions and condition of the accessory sex glands, epididymis and testis were evaluated with the use of ultrasonographic imaging (Figs. 1 and 2). The volumes of the accessory sex glands (Table 4) were significantly different between the three semen categories. The volumes of these glands (the bulbourethral gland,

Table 4
Volume of accessory sex glands in adult male white rhinoceroses with different semen category

Semen category	Animals	Bulbourethral gland volume (cm^3)	Prostate volume (cm^3)	Seminal vesicle volume (cm^3)
I	12	32.6 ± 1.8[a]	50.4 ± 17.4[a]	46.4 ± 5.4[a]
II	5	12.1 ± 1.6[b]	21.8 ± 2.9[a,b]	31.2 ± 3.6[a,b]
III	5	11.3 ± 1.2[b]	19.5 ± 1.5[b]	30.2 ± 3.6[b]

The columns with different superscripts (a, b) are significantly different (Dunn's multiple comparisons test).

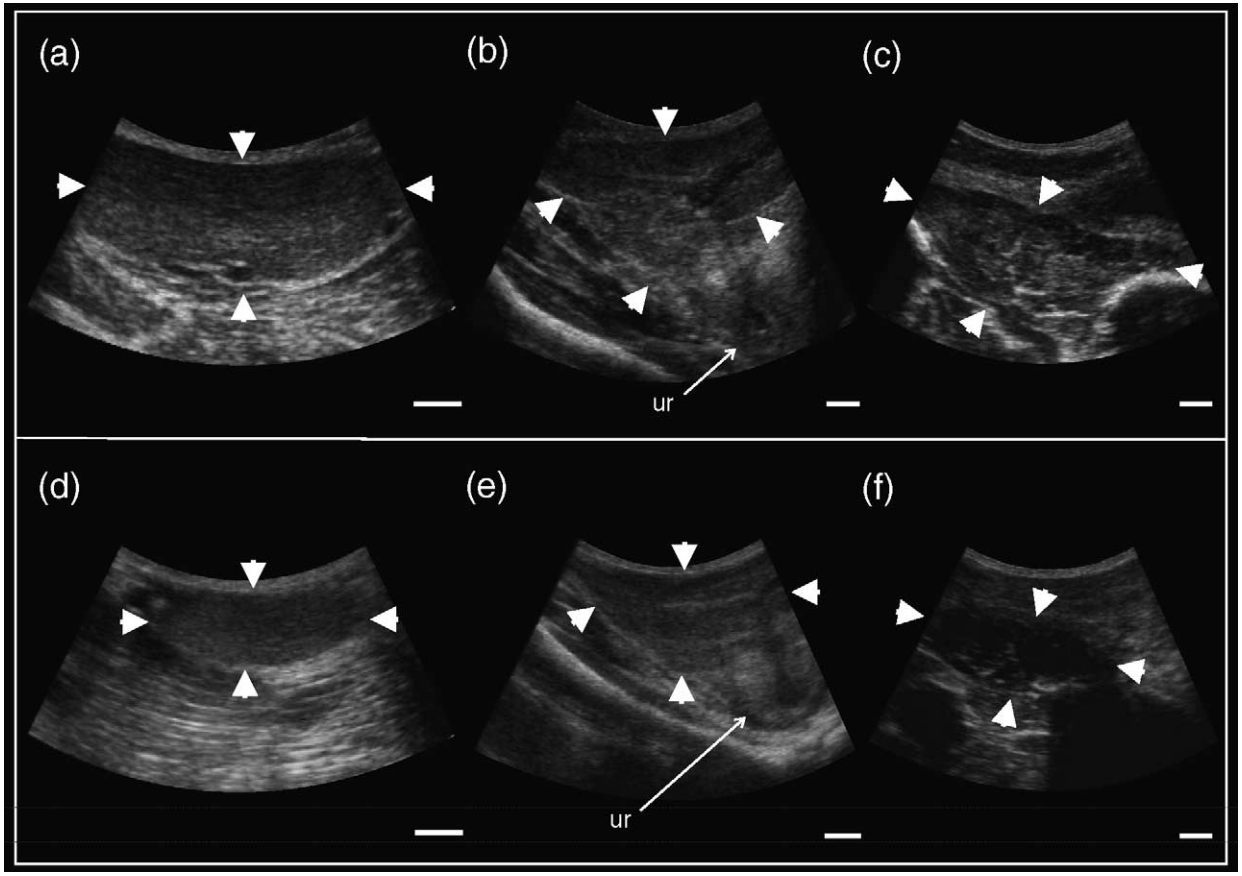

Fig. 1. Sonograms of the bulbourethral gland (a, d), the prostate ((b, e) and the seminal vesicle (c, f) in male white rhinoceroses with high (a–d) and low semen quality (d–f). ur: urethra in cross-section. Note the difference in gland size between males with different semen quality. Scale bars represent 1 cm.

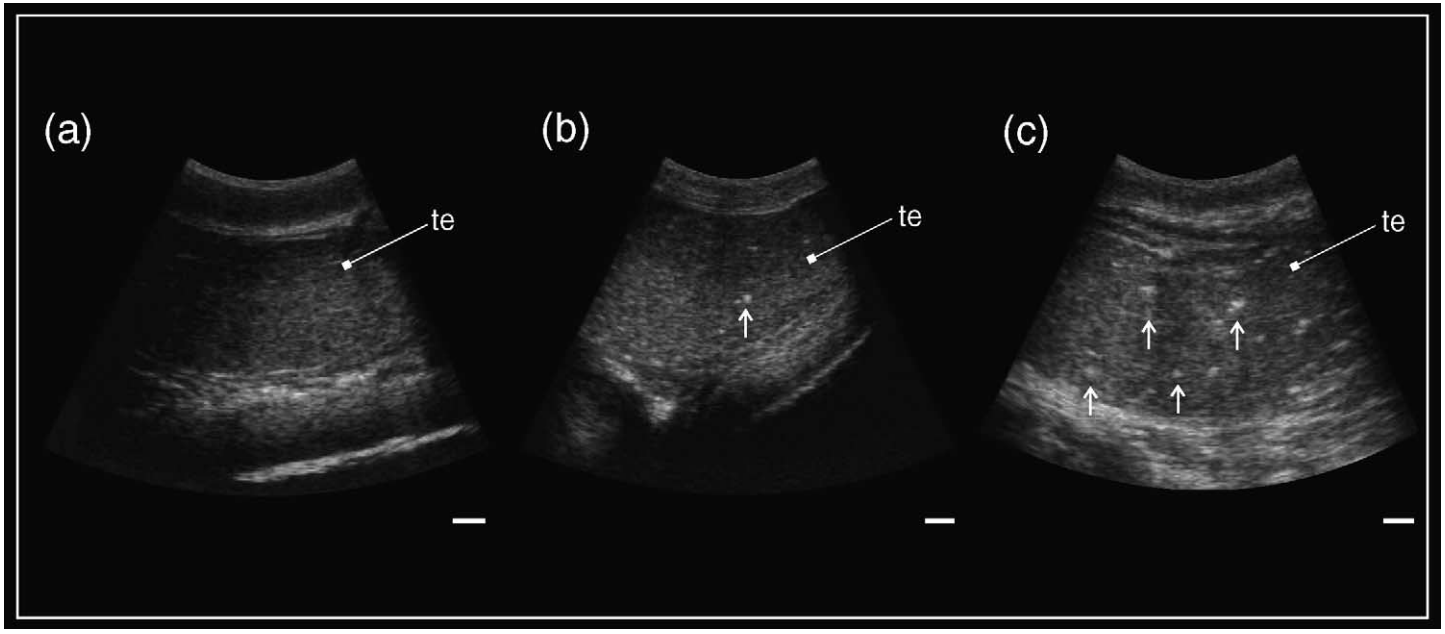

Fig. 2. Sonograms of the testis of three adult male white rhinoceroses. te: testicular parenchyma. (a) Homogenous testicular parenchyma in a 13-year-old male (No. 974). (b) A single foci of fibrosis in the parenchyma in a 20-year-old male (No. 770). (c) Multiple foci of fibrosis distributed throughout the testicular parenchyma in a 30-year-old male (No. 348). Scale bars represent 1 cm.

Table 5
Spearman rank coefficients between volume of accessory sex glands and spermatological parameters in white rhinoceroses

Parameter	Bulbourethral gland	Prostate	Seminal vesicle
Sperm motility	0.812***	0.441**	0.312*
Intact sperm	0.604***	0.443**	0.392*

The asterisks indicate the statistical level of significance.
* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.0001$.

the prostate and the seminal vesicle) were positively correlated with the percentage of motile and intact spermatozoa (Table 5).

Based on ultrasound the mean testis and epididymis diameter was 6.5 ± 0.3 cm and 2.8 ± 0.1 cm ($n = 24$), respectively. In the testis, hyperechoic spots characterized a focal fibrosis of the testicular parenchyma in males ≥15 years of age. The occurrence of multi-focal testicular fibrosis was higher in older males (≥30 years) compared to middle-aged males (15–30 years) and, young adults (8–10 years) with no detectable hyperechoic spots (Fig. 2). Testicular fibrosis was not found to affect the ejaculate parameters in the white rhinoceroses since three of the oldest males (No. 1; 160; 363) with advanced fibrosis consistently showed high semen quality ($n = 6$) (total number of sperm: $(4.1 \pm 2.1) \times 10^9$, sperm motility: 82.5 ± 4.7%, morphological integrity. 62.8 ± 7.3%). Age related atrophy or atrophy due to breeding inactivity of the testis was not detected in any of the rhinoceroses.

Previous post mortem ultrasonographic and histologic examinations of the testis from two isolated genital tracts have shown the occurrence of diffuse testicular fibrosis in the rhinoceros. Macroscopically, the testicular parenchyma was intersected by multiple broad white, firm streaks distant to the rete testis. Histological sections of these areas (not shown) revealed numerous fine to coarse bands of mature connective tissue separating the seminiferous tubules from each other. There was no evidence of inflammation. The intact, moderately active germ cell layers with some intraluminal, mature spermatozoa lined the seminiferous tubules. Histology validated ultrasonographic findings, which had shown multiple hyperechoic spots throughout the testicular parenchyma in these males.

3.3. Semen quality compared with male group size

In order to determine whether social structure influences reproductive function in the white rhinoceros, categorized ejaculates were compared with the group sizes in which male rhinoceroses were managed (Table 1). Four different group settings were evaluated. In a multi-male ($n = 5$) group housed in one large enclosure (0.7 km^2) with six females, only the territorial and dominant male (No. 487) showed good semen quality (category I). All other males showed intermediate or low semen quality. Following the first assessment of these five males, the management of the multi-male group was changed. One formerly subdominant male with low semen quality was granted his own territory, undisturbed by the presence of other male, with exclusive access to cycling females. The semen quality of

Table 6
Semen characteristics of a subdominant white rhinoceros in a multi male group (Atari, No. 497, ejaculate 1) and after establishment of an own single male territory with exclusive access to cycling females (ejaculate 2)

Parameter	Ejaculate 1, April 2001	Ejaculate 2, May 2003
Group size (m/f)	5.6	1.2
Volume (ml)	71	8.5
Sperm conc. (10^6/ml)	11.94	150
Total sperm (10^9/ejaculate)	0.848	1.275
Sperm motility (%)	50	90
Intact sperm (%)	60	82
Semen category	III	I

this male had improved from the low to the high category upon re-evaluation, 6 months after the management changes were implemented (Table 6). This animal subsequently bred and sired offspring.

In facilities ($n = 5$), housing two males with three to five females, only one male showed high semen quality while the other male showed either intermediate or low semen quality. Three of six males housed as a single territorial bull with one to three females had high semen quality. Males ($n = 4$) housed individually without any accompanying male or female showed high semen quality.

3.4. Preservation of spermatozoa by chilling for 24 h

Chilling results of the BC-extended semen during a 24 h period at 4 °C revealed that white rhinoceros spermatozoa were not sensitive to cooling as mean sperm motility ($n = 7$) before and after 24 h of chilling remained almost constant (80.7 ± 9.1% and 77.1 ± 6.2%) ($P = 0.743$).

3.5. Cryopreservation of spermatozoa

Fourteen ejaculates from 11 Southern and 1 Northern white rhinoceros were frozen in liquid nitrogen using a custom-made cryoextender (BC), in order to further establish methods of cryopreservation and genome resource banking in the white rhinoceros. Mean post-thaw motility with this cryoextender was 32.8 ± 3.7 (range: 20–50%) compared with

Table 7
Comparison of post thaw sperm motility in ejaculates from five southern white rhinoceroses using four different cryoextenders

Cryoextender	Sperm motility before freezing (%)	Sperm motility post thawing (%)	Sperm motility maintained (%)
Native semen	57.0 ± 4.4	–	–
Berlin	62.6 ± 4.4	27.6 ± 3.4	49
Biladyl	53.0 ± 4.4	25.2 ± 3.5	44
Kenny modified	57.0 ± 4.6	24.6 ± 5.6	44
Gent	47.0 ± 7.0	17.5 ± 9.7	32

the mean native motility of 67.9 ± 4.2% and pre-freeze motility of 71.9 ± 3.3%. In a further comparative study, five ejaculates using BC and three commercial cryoextenders (Table 7) demonstrated that BC maintained sperm motility before freezing on average 5% above cooled unextended semen. Biladyl and Gent extender showed a decrease in sperm motility before freezing by 5% and 10%, respectively. After thawing, the three extenders, BC, Biladyl and Kenny, showed similar post-thaw motility results ranging from 25% to 28%.

4. Discussion

To date, the reproductive status of male white rhinoceroses has scarcely been evaluated. This is mainly due to anaesthesia risks [16] and to the technical difficulties involved with fertility assessment of male white rhinoceros. The provided information about the reproductive status of 21 captive white rhinoceroses represents a substantial physiologic database on reproductive parameters in this species. The correlations discovered between the volume of the male accessory sex glands and the associated proportions of motile and intact spermatozoa suggest that reproductive fitness requires both sufficiently developed accessory glands and adequately stimulated spermatogenesis. Regulation of reproductive fitness seems to be influenced by the number of males in a social group and social status of the male. This conclusion is supported by the single case where the social status of one male was changed. The result was a clear increase in sperm quality. Additionally, methods for semen preservation and cryopreservation were assessed and these techniques may aid in future assisted reproduction programs for rhinoceros.

Ultrasonography was confirmed as a valuable tool to assess the reproductive health status and to detect potential disorders of the reproductive organs. Testicular fibrosis was the only abnormal finding detected and was common in mid-aged as well as older white rhinoceroses. The association between the size and number of fibrotic spots in the testicular parenchyma and the age of the rhinoceros illustrated that the onset of testicular ageing was at approximately 15 years of age with age-related progression of fibrosis. Degenerative age-related changes associated with the germ cell degeneration have been reported in several other mammals such as human, mouse, horse and the Indian rhinoceros [23–26]. For example in stallions ageing of the reproductive organs manifests in progressive testicular degeneration and reduced sperm output, compromised libido and mating ability [27]. However, in all white rhinoceroses older than 30 years, semen quality was high. It could not be determined whether the sperm output of these animals had changed over time because prior examinations had not been performed. A compromise of libido and mating ability due to a reproductive ageing process cannot be ruled out as a contributing factor to the lack of fecundity in older male rhinoceroses.

Various methods of semen collection have been described for rhinoceros. Manual semen collection, sometimes supported by detomidine-HCl and butorphanol, represents a method established in a few individuals from a variety of species [9,10,28]. However, this method of semen collection is time consuming, has limited success and requires intensive animal training. Post coital sperm collection, as described in the Sumatran rhinoceros, is the least invasive method for sperm evaluation and preservation. Success by this method remains, at this point, limited to one animal [29].

In this study, electroejaculation applied to the specific anatomical structure of interest, as visualized by ultrasound, proved to be a reliable method of semen collection. Electroejaculation was applicable to all rhinoceroses regardless of their training standard or husbandry. Electroejaculation resulted in the recovery of larger ejaculatory volumes when compared with manual stimulation or previously reported electro-stimulation results in the black rhinoceros [10,28]. This suggests that the use of the adapted electro-stimulation probe allowed an improved stimulation of the accessory sex glands and consequently a more complete ejaculate collection. General concerns with these results presented here are that much of the data are based on only one ejaculate per animal representing only one data point. Despite the collection of only one ejaculate, most of these animals masturbated frequently. Consequently the semen collection by electroejaculation did not represent the first ejaculation. Nevertheless, the extrapolation of the data to the entire population has to be cautious.

Captive white rhinoceroses exhibited a large range of semen quality. The categorization of semen quality based on the proportion of progressively motile spermatozoa is simple, but reliable, since the motility is one of the important prerequisites of the fertilizing capacity. The domestic horse, the closest related domestic species to the rhinoceros, is widely regarded as a model species in male [14,26,29] and female rhinoceros reproduction [1,4,14,30,31]. Stallion semen preservation protocols and cryodiluents have specifically been used in three rhinoceros species, including the white rhinoceros, demonstrating the validity of the horse model [32,33]. In the stallion, sperm motility and morphology are positively correlated with pregnancy rate [34,35] and are regarded as best prognostic indicator for fertilization in vitro [36] and in vivo [37]. Therefore, we used similar motility levels for the high and lower semen quality categories as those used in stallions [38,39]. Two rhinoceroses with intermediate and low semen quality had reproduced in the past. We assume that the fertilizing potential including libido and sperm parameters of these males changed over time. Overall only 52% of the males showed high sperm quality (category I) although all males had access to reproductively healthy females (Hermes, unpublished). This suggests that reduced semen quality could be a contributing factor for the low rate of reproduction in the captive white rhinoceroses.

The high percentage of white rhino with reduced semen quality may be attributed to the artificial social structure created in captivity. In the wild, single dominant males divide land up into a mosaic of single male territories averaging 1.65 km^2. Territorial males tolerate other bulls in their territory only if they display clear gestures of subordination. In the wild, subordinate bulls within the territory of an alpha bull undergo behavioural changes, and are prevented from mating females within the group [40]. This behavioural subordination has also been reported to occur in captivity [15]. Our results showed that only a single male in a two or multi-male facility showed high semen quality. The other socially subordinated males displayed decreased reproductive fitness in the presence of the territorial male as indicated by reduced semen quality and reduced volume of accessory sex glands. In contrast to a two or multi-male group, all rhinoceroses housed solitarily in a facility without accompanying female showed consistent high semen quality representing unchallenged territorial bulls.

The influence of social organization on reproductive behaviour has been shown in elephants and wild horses [41,42]. Confined multi-stallion breeding farms create artificial

bachelor/subordinate status and subsequently reduced reproductive function (poor or intermittent semen quality). Altering the captive social structure from an artificial bachelor herd composition to the natural harem structure resulted in improved reproductive parameters in stallions [41]. In a case study involving management adjustments in a multi-male rhinoceros facility, improved semen parameters, breeding behaviour, and confirmed pregnancy were seen, similar to that seen in stallions, due to the upgrading of a subordinate male to a territorial status (A. Terkel, personal communication). The finding that all solitary housed males had high semen quality suggests that males do not need the female presence to produce good quality ejaculates. Reasons for low semen quality in single males housed with one or multiple females remains speculation and needs further investigation. Long-term (10–15 years) sexual rejection in the presence of a territorial male might be one cause for poor reproductive parameters in these males. In general we conclude that better understanding of territorial needs of males in relation to other males and females is an important aspect for captive breeding management and needs further research.

White rhinoceros are not described as seasonal breeders. Although in the wild, a peak in number of conceptions and in the level of serum testosterone was reported at the start of the rainy season [40,43–45]. This peak is most probably caused by a nutritional flushing effect when fresh grass first becomes available [40]. In captivity, rhinoceroses are not exposed to extreme nutritional imbalances throughout the seasons, therefore, the flushing effects might not occur. Furthermore, rhinoceros' diets and vitamin and mineral supplements, including Vitamin A in captivity exceed their nutritional needs ([46], Clauss, unpublished). However, over the last 10 years beta-carotene has been recognized not merely as a pro-Vitamin A but as a substrate which directly regulates reproductive processes. Studies on the possible role of beta-carotene on male reproduction are lacking [47]. Preliminary data (Hermes et al., unpublished) suggest that beta-carotene supplementation in male rhinoceros might improve sperm quality when supplemented over a long period of time.

During this study semen from nine non-reproducing southern and two northern white rhinoceroses was cryopreserved. For cryopreservation, we used a cryoextender (BC), which was developed and applied in attempts to cryopreserve semen of several endangered species [19]. Cryoinjury of spermatozoa is caused during cooling and freezing by interacting biophysical and biochemical changes within the cells and within the environment [48,49]. The lowering of temperature before the freezing point effects the equilibrium between the intra- and extra cellular solutes, which depend on active transport mechanisms. The cells pass through different quasi-stationary states according to different time constants of water, anion and cation exchange, as described in a model for the osmoregulation of erythrocytes [50]. This model demonstrated, that colloidosmotic stress in these transient stages decreases because of the balance between electrolytes and impermeable non-electrolytes in the solution. We considered this osmoregulatory model in our basic buffer used for the cryopreservation.

Cryopreservation of epididymal sperm from culled white rhinoceroses or of post coital sperm from a Sumatran rhinoceros has previously demonstrated the feasibility of cryopreservation in three rhinoceros species ([29,32,33]; Schaffer, personal communication). In this study, it was demonstrated that gametes from a large number of non-represented male rhinoceroses can be accessed in vivo, preserved, transported, stored as the genetic reserve and used in potential assisted reproduction programs. These results can be of interest

to conservation efforts of the Northern white rhinoceros, which is considered one of the rarest large mammal subspecies on earth. There has been much concern about the status of the last surviving wild population of only 30 animals in the Republic of Congo because of recent civil wars and lack of supervision [51]. Cryopreservation of sperm from one captive Northern white rhinoceroses in this study represents an important pre-requisite for additional conservation efforts of this critically endangered subspecies. As the genetic diversity is diminished to such low numbers, the success of in situ management becomes increasingly important. Although, first priority in the conservation of any species is to ensure the protection of the in situ population, gamete cryopreservation by means of electroejaculation, customized semen extenders and freezing technology may facilitate the recovery and storage of semen from selected wild males as a reservoir of genetic material to be used to improve development of a reserve population without reducing the viability of the wild one.

For the comparative study of different cryoextenders only intermediate and low semen quality ejaculates were available. Therefore, post thaw motilities of 24–28% were below the results from higher quality ejaculates (20–50%) in which BC exclusively had been used. However, different from the other cryodiluents tested, the BC seemed to enhance sperm motility before freezing and after thawing. This is below the level of significance and must be confirmed with greater number of samples.

In summary, the presented study provides a database on reproductive parameters of captive male white rhinoceros. The collection of comprehensive data and the execution of experimental studies with a large number of animals are limited by the nature of the rhinoceros and their husbandry in captivity. Therefore, some of the reported data are not statistically significant. However, we consider this information as relevant for further improvement of captive breeding programs.

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