

Technical aspects of improved semen collection procedures in African rhinoceroses (*Ceratotherium simum*; *Diceros bicornis*) under field conditions

J Meuffels-Barkas,^{1,2} I Luther-Binoir,^{1,3} H Bertschinger,² I Callealta,⁴ B Tindall,⁵ I Lueders^{1,6}

¹Cryovault, Hemmersbach Rhino Force SA NCP, South Africa

²Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, South Africa

³GEOSperm, Wildlife Reproduction and Biotechnology Services, South Africa

⁴ECOLifes, Animal Reproduction and Ultrasound Services, Spain

⁵Robberg Veterinary Clinic, South Africa

⁶Mammal Research Institute, South Africa

Corresponding author, email: meuffels.janine@gmail.com

Wild rhinoceros populations are threatened by ongoing poaching pressure and habitat loss. Establishing reservoirs of gametes of as many individuals as possible may assist to preserve genetic diversity and could be applied in assisted reproductive techniques in the future. However, for routine implementation of semen collection during any male rhinoceros immobilisation event, current methods and protocols need simplification and improvement.

This study aimed to establish field-friendly methods of semen collection in white (*Ceratotherium simum*; WR) and black rhinoceroses (*Diceros bicornis*; BR) that can be performed opportunistically given the usual time constraints.

Sixty-two game-farmed WR and seven wild BR were immobilised for planned management interventions. Semen collection was performed using electro-stimulation with a battery powered electro-ejaculator and specifically designed rectal probe. Furthermore, in ten WR and two BR, urethral catheterisation was performed with a 10FG nasogastric tube passed retrograde up the urethra as a method of semen collection.

Semen-rich fractions were collected from 47/62 WR and 6/7 BR following electro-stimulation. Of these, 10 WR and two BR samples were obtained by urethral catheterisation. The duration of semen collection was 16.4 ± 7.3 (3–30) and 25.8 ± 6.0 (19–33) min requiring 5.2 ± 2.3 and 6.0 ± 0.7 stimulation sets for WR and BR, respectively. Semen volumes, sperm concentrations, motile and viable sperm were 1–64 and 9–18 ml, 68.4 ± 54.2 and $23.7 \pm 26.7 \times 10^6/\text{ml}$, 51.0 ± 14.2 and 55.0 ± 17.3 , and 79.9 ± 15.0 and $88.3 \pm 8.4\%$ for WR and BR, respectively. Urethral catheterisation, when implemented, reduced the time required for semen collection. Operator experience influenced sampling efficacy.

Equipment and methods described in this study improved semen collection in free-ranging rhinoceroses and can be combined with routine immobilisations, despite time constraints.

Keywords: black rhinoceros, electro-ejaculation, sperm, urethral catheterisation, white rhinoceros

Introduction

Besides environmental pressures, including habitat degradation and fragmentation, and human-wildlife conflict (Milligan et al. 2009), African rhinoceros populations are facing a continual high threat of poaching for the illegal horn trade (Emslie 2020a;b). As a consequence, the black rhinoceros (*Diceros bicornis*) is categorised as “critically endangered” by the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (Emslie 2020a). The Southern white rhinoceros (*Ceratotherium simum simum*) is considered “near-threatened” despite massively declining numbers, with latest estimates painting a bleak picture (Nhleko et al. 2021).

Conventional conservation efforts largely focus on habitat protection, reintroductions, anti-poaching measures and captive breeding programmes. More recently, the use of assisted reproductive technologies (ART) (Prieto et al. 2014) and genetic biobanking (Ballou et al. 2023) have been advocated as additional tools for the conservation of endangered and threatened species to curb further losses of genetic diversity.

Due to the Northern white rhinoceros being declared as functionally extinct recently, international efforts have increased to advance lab-based ART for this subspecies (Hermes et al. 2018; Hildebrandt et al. 2018). However, only limited amounts of biomaterial from a handful of individuals have been cryopreserved. Therefore, starting ART efforts only now appears hopeless from a practical and genetic point of view for this subspecies.

More than ever, in the light of rapidly declining numbers of African rhinoceros, semen collection and cryopreservation are an essential component for bio-banking of viable genetic material (Hermes et al. 2018). For successful application of frozen-thawed semen in artificial insemination (AI) or in vitro fertilisation (IVF), collection of good quality sperm samples is required. Semen collection and assessment is also part of any breeding-soundness evaluation in captive breeding programmes. Electro-ejaculation (EE) under chemical immobilisation is the most commonly used method to obtain ejaculates in non-domestic species (Prieto et al. 2014).

For captive and free-ranging white rhinoceroses, reports of successful semen collection via electro-ejaculation are limited (Hermes et al. 2005; Luther 2016; Roth et al. 2005). In black rhinoceroses, reports are restricted to small numbers of captive individuals (Hermes et al. 2018; Roth et al. 2005).

Apart from technical details, procedural aspects are important if results are to be improved. For captive rhinoceroses under controlled conditions, where lateral recumbency for collection was used, anaesthesia and monitoring of animals is possible for extended periods (Hermes et al. 2005, 2018; Roth et al. 2005). In free-ranging rhinoceroses, however, sternal recumbency is preferred as respiratory depression associated with etorphine-induced immobilisation, is exacerbated in lateral recumbency (Morkel et al. 2010). Additionally, the anaesthesia down-time of wild specimens should be restricted, meaning that protracted procedures are contraindicated. Semen collection under free-ranging conditions is further complicated by different in-the-field settings, such as climate and terrain. To enable successful and safe semen collection under free-ranging conditions, field-friendly equipment and protocols are required (Arnold et al. 2017).

Today, management interventions such as dehorning, collaring and translocation of wild rhinoceroses have become an essential part of conservation efforts in Southern Africa. Simultaneously, these events offer opportunities to access males for semen sampling if performed time- and cost-efficiently.

Thus, the aim of this study was to develop and test portable field-friendly equipment, including a battery powered electro-ejaculator with custom-designed rectal probe, for semen collection under time constraint (< 30 min). To improve the overall in-the-field application of EE in rhinoceroses, factors such as operator experience, positioning of the animal, and influence of immobilising drug combinations used, were evaluated. Additionally, the suitability of urethral catheterisation (UC) for semen collection in rhinoceroses, a method established for several carnivore species (Jeong et al. 2018; Lueders et al. 2012) and recently described in captive black and white rhinoceroses (Moresco et al. 2024) was investigated. Results aim to encourage the incorporation of semen collection into routine immobilisations of (African) rhinoceros species for management purposes.

Research methods and design

Animals and study areas

Sixty-two game-farmed Southern white rhinoceroses (*Ceratotherium simum simum*; WR) and seven free-ranging Southern black rhinoceroses (*Diceros bicornis minor*; BR) were immobilised for planned management procedures between June 2020 and March 2021. These events allowed for opportunistic semen collection of up to 20 WR per week.

Only physically healthy, sexually mature males (WR: 12.57 ± 5.92 [5–30] years; BR: 15.83 ± 8.42 [7–28] years) were included in this study. WR were located in managed camps (approx. 500–700 ha each) on game farms in Limpopo and the Northern Cape provinces of South Africa; BR in a public wildlife reserve in the

Eastern Cape. Of the WR, 21 bulls had access to females, while 41 were kept in male-only groups (25–35 bulls per camp).

The study was approved by the Animal Ethics and Research Committees (protocol REC113-19) of the University of Pretoria.

Capture and immobilisation

Black and white rhinoceroses in this study were anaesthetised as published previously (Meuffels et al. 2022, 2021). Briefly, the 49 WR and seven BR received 4–6 mg etorphine (etorphine hydrochloride 9.8 mg/mL, Captivon; Wildlife Pharmaceuticals, Karino, South Africa) combined with 4–5 mg medetomidine (20 mg/mL, Kyron Laboratories, Benrose, South Africa). The remaining 20 WR received 2–3 mg etorphine and 10 mg medetomidine. Additionally, 50 mg midazolam (midazolam hydrochloride 50 mg/mL, Dazonil; Wildlife Pharmaceuticals) and 1500–2500 IU hyaluronidase (lyophilised hyalase, Kyron Laboratories) were included in each induction drug combination.

Immobilisation drugs were delivered remotely by dart gun (Pneu-Dart 389, Pneu-dart, Inc., Williamsport, Pennsylvania, USA) using 3.0 mL Pneu-dart darts (Pneu-dart Type C, Pneu-dart, Inc.) with 2.5-inch uncollared needles into the gluteal muscle-mass or nuchal hump from a helicopter (BR and WR in Limpopo) or on foot (WR in the Northern Cape). WR anaesthetised with higher doses of etorphine (4–6 mg) and medetomidine, received 5 mg butorphanol (50 mg/mL; Kyron Laboratories) intravenously into an auricular vein at first handling. Once recumbent, the animals were blindfolded, and cotton wool earplugs were applied. For semen collection, WR were positioned either in sternal ($n = 42$) or lateral ($n = 20$) recumbency, whereas all BR were positioned sternally.

Anaesthesia was reversed with an intravenous or intramuscular combination of 50 mg naltrexone HCL (Trexonil 50 mg/mL, Wildlife Pharmaceuticals) and 10–20 mg atipamezole HCL (5 mg/mL mg; Atipamezole, Vtech, Midrand, South Africa or Antisedan, Zoetis, Sandton, South Africa depending on availability).

Ultrasound examination

Faeces were removed manually from the rectum using a lubricated rectal glove. This was followed by a rectal enema of 1–2 L water administered with a 20 mm polyethylene tube connected to a 10 L jerry can. Trans-rectal ultrasound (US) examination of the accessory sex glands was then performed using a portable, battery-powered US machine (Fujifilm Sonosite Inc., Amsterdam, Netherlands) with a hand-held convex transducer (C60xi, 2–5 MHz). The prostate, seminal vesicles, and bulbourethral glands were located to assist with correct placement of the electro-ejaculator probe.

Semen collection

Electro-ejaculation probe design

Semen collection was performed using a portable, battery-powered electro-ejaculator (El Toro 3, Electronic Research Group, South Africa) and custom-made, rhinoceros-specific probe with electrodes (Figure 1) designed according to the reproductive anatomy of rhinoceros (Electronic Research Group, South Africa). The smooth material (Polyurethane resin and PVC), cylindrical



Figure 1: Custom-made rhinoceros electro-ejaculator probe with external handle and three slightly raised electrodes on the ventral surface of the probe head. A = dorsal view; b = ventral view; c = lateral view.

shape and rounded front of the probe, allow for easy and non-traumatic insertion into the rectum. Three, longitudinal, slightly raised stainless steel electrodes are positioned on the ventral aspect of the probe. The length of the electrodes (8.5 x 1.8 cm with 1.8 cm space between the electrodes) is based on length of the pelvic urethra and the area to be stimulated caudal to the urinary bladder. The large probe diameter (17 x 11.5 cm) provides good contact between the electrodes and the rectal mucosa. The long handle attached at an upward angle in relation to the electrodes was designed to allow adequate external manipulation and positioning of the probe with animals in sternal position (Figure 2).

Electro-ejaculation (EE)

The EE procedure ideally required three people: one person to place and hold the rectal probe, a second to operate the electro-ejaculator and a third to hold the penis and change semen capture sleeves.

After the US assessment, and prior to insertion of the EE probe, the prostate and surrounding area were massaged manually trans-rectally to facilitate relaxation of the penis for manual extrusion from the prepuce. Once extruded, the glans penis was cleaned with gauze swabs and physiological saline. During electro-ejaculation, the semen was collected in 50 mL insulated polypropylene conical tubes (Plastpro Scientific [Pty] Ltd, 6 Wakefield Rd, Founders Hill, Lethabong, 1609, South Africa) attached to a funnel made from a plastic rectal sleeves, which was placed over the tip of the penis (Figure 3b). To allow semen fractions to be collected separately, the collection sleeves with tubes were changed at regular intervals and, at least, after each set of stimulations.

The well-lubricated EE probe was inserted in a slightly upward direction into the rectum and positioned dorsal to the approximate position of the prostate, with the electrodes facing ventrally (Figures 1–3). The ventral aspect of the rectal mucosa was palpated for the presence of folds, which if present, were stretched. The position of the probe was secured throughout the stimulation procedures by holding the handle in one hand and placing the other on the dorsal surface. To avoid possible urine

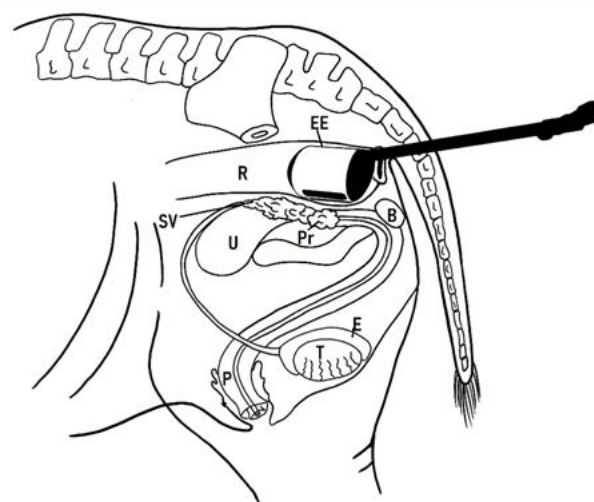


Figure 2: Schematic presentation of the anatomy of the reproductive system of the male rhinoceros with the correctly positioned electro-ejaculator (EE) probe; P = penis, Pr = prostate, B = bulbourethral gland, E = epididymis, R = rectum, SV = seminal vesicles, T = testis and U = urinary bladder.

contamination during stimulation, care was taken not to allow the probe to move cranially over the bladder neck.

Once the probe was in position, stimulation commenced. If retraction of the penis or hind leg muscle contractions occurred, the probe was repositioned until leg movements ceased and the scrotum lifted upon stimulation. Contractions of the urethra and scrotum in the perineal region during stimulation indicated correct placement. Electric stimuli were applied in sets, each consisting of 8–10 stimulations lasting 2–3 seconds, with 2 second pauses in between. Following each set of stimulations, the prostatic area and penile urethra (the latter in the direction of the glans penis) were massaged. The voltage was slowly increased with each set to reach a maximum of 10 V and 277 mA. At the end of the set that produced a semen rich fraction, 5–8 consecutive higher voltage (8–10 V) bursts were applied in an attempt to eject all fluids that may have accumulated in the urethra.

Collection tubes containing ejaculates were immediately transferred to an insulated box with a temperature of 18–23 °C and, depending on environmental temperature, maintained by either adding bottles filled with warm water or cooling pads. If urine contamination occurred, the procedure was immediately aborted.

Urethral catheterisation

UC was tested in seven WR and two BR under the following circumstances: 1. anaesthesia had to be reversed, and 2. after several electro-stimulation sets no sample had been obtained. A 10FG, 3.3 mm x 100 cm flexible nasogastric tube (DynaMed Pharmaceuticals [Pty], Durban, South Africa) with a smooth, closed front and rounded tip, resembling urinary catheters developed for small domestic animals, was introduced up to 60 cm into the urethra of the extruded penis after applying a non-spermicidal sterile lubricant (Figure 3). Additionally, to accommodate the length and diameter of the rhinoceros penis, an 0.66 x 137 cm equine urinary catheter (Jorensen Laboratories,

Inc., Loveland, CO, USA) was tested. Semen, if present, was allowed to flow passively down the catheter. If no semen was present in the lumen upon retraction, the catheter was inserted a second time. Intermittent negative pressure was applied and maintained with a 10 ml syringe prior to or during slow retraction of the catheter.

Semen evaluation

The volume, colour and consistency of the sperm-rich fractions were recorded at the time of collection. Microscopic evaluation was carried out in a field laboratory. The delay between semen collection and evaluation was subject to the distance between site of collection and the field laboratory. Most evaluations were carried out within 15–30 min of collection, however in four WR and one BR, evaluations were performed 60 to 145 min after collection. Microscopic evaluations were carried out using phase contrast and bright field microscopy. A 10 μ L aliquot placed on a pre-warmed slide (37 °C) was used to subjectively assess motility. Motility was expressed as % motile (total motility) and % progressively motile sperm. Sperm concentration was assessed with a Neubauer haemocytometer. Sperm morphology, viability and acrosomes were evaluated in a dried eosin-nigrosin thick-thin smear (McGowan 2018) and recorded as % normal, % viable sperm and % intact acrosomes, respectively.

Semen samples with very few or no motile sperm and samples contaminated with urine (based on visual inspection and smell) were discarded and excluded from statistical analyses. All other samples were further evaluated and categorised as “good” ($\geq 45\%$ for each of the following parameters: % motile sperm, % normal sperm, % intact and % viable sperm and $> 25 \times 10^6$ sperm/mL) or “poor” (one or more of the before mentioned parameter below 45%).

Data analyses

Statistical analysis was performed with PSPP (Gnu Project 2015). Data were assessed for normality by calculating descriptive statistics, plotting of histograms and using a Shapiro-Wilk test. Data was not normally distributed ($p = 0.00$), therefore non-parametric tests were used. Median (range) was calculated for each semen parameter per species (Table I). Mean $\pm$ SD were included to enable a better comparison with previously published reports. The frequency of successful semen collection (%; sperm rich fractions with motile sperm) and incidence of urine contamination (%) were calculated for all collections. Additionally, the successful semen collections (%) and urine contaminations (%) of the first 25 and later 37 collections were compared to determine the effect of operator experience which was expected to improve over time and included positioning of the rectal probe (operator 1) as well as the electro-stimulation procedure (operator 2). In WR, semen quality variables were compared between samples collected via EE and UC and between bulls with different social statuses (housed with females vs. housed without females) using a Mann-Whitney U test. A possible correlation between social status, animal position (lateral vs. sternal recumbency) during collection, and anaesthetic protocol (high vs. low medetomidine dose) and the chance of successful semen collection in WR was evaluated using

a logistic regression. Differences were considered significant when $p \leq 0.05$.

Results

All animals were successfully anaesthetised and standing within three minutes of administration of reversal drugs. No adverse physical or behavioural effects, during the procedure or after the recovery period could be associated with semen collection.

Semen collection

Sampling methods

The custom-built EE probe allowed easy rectal insertion and manipulation. Semen-rich samples were collected from 47 WR (76%) and six BR (85%). Seven and two of these samples were partly or exclusively obtained by means of UC following electro-stimulation in WR and BR, respectively (Table I).

Electro-stimulations commenced 27.45 ± 11.69 (8–56) min in WR and 11.67 ± 3.98 (7–17) min in BR after darting. The duration of semen collection, from start to completion of stimulation or UC, was 16.4 ± 7.3 (3–30) and 25.8 ± 6.01 (19–33) min and the number of stimulation sets was 5.27 ± 2.3 and 6.0 ± 0.7 for WR and BR, respectively.

UC in rhinoceroses was altogether successful when performed with a 10 FG nasogastric tube. When using the larger diameter equine catheter, spontaneous capillary semen flow was not observed. The application of negative pressure using a syringe was required and resulted in low volume samples containing large numbers of epithelial cells, which were likely caused by suction trauma to the urethral mucosa. UC helped to shorten the duration of semen collection as further stimulations to achieve semen emission were not required. In two WR, duration of semen collection was shortened to less than 10 min. In two WR and one BR, where no ejaculation occurred after 20–30 min of electro-stimulation, UC semen samples were obtained.

Operator experience

The success rate of semen collection following EE in WR, increased with the operator experience from initial (first two large scale sampling events; 14/25; 56%) to later (33/37; 89%) collections, respectively ($p = 0.01$). In WR, the incidence of urine samples or urine contaminated samples prior to a sperm rich fraction was 16% (10/62). Although not statistically significant ($p = 0.8$), the incidence decreased from 20% (5/25) initially to 14% (5/37) during later samplings. More caution was taken to position and keep the probe as far caudally as possible (right up against the anal sphincter) during stimulations in the later collections.

Effects of medetomidine dose and recumbency position

In WR, the highest success rate of semen collection was achieved in animals immobilised with 10 mg vs. 5 mg of medetomidine (18/20; 90% vs. 28/42; 67%; $p = 0.05$). Higher doses of medetomidine also lowered incidence of urine contamination, but the difference was insignificant (2/20; 10% vs. 8/42; 19%; $p = 0.3$). However, neither the anaesthetic protocol nor recumbency position was associated with a significant



Figure 3: a: Semen collection using electro-ejaculation in a free-ranging black rhinoceros in sternal recumbency. The probe is inserted into the rectum with external handle facing upwards and connected to a portable, battery-powered electro-ejaculator. b: Placement of the semen capture sleeve over the tip of the extruded white rhinoceros penis in sternal recumbency. c: Urethral catheterisation for semen collection in a black rhinoceros using a lubricated, soft, flexible 10FG nasogastric feeding tube.

improvement in semen collection ($p = 0.09$ and $p = 0.41$, respectively).

Semen quality

Rhinoceros semen samples varied from white to light grey in colour and a milky to watery consistency. Thirty-six (56%) WR and four (57%) BR produced samples suitable for further evaluation. Of these, 15 (24%) and three (43%) had good quality, respectively.

Semen samples with very few or no motile sperm (WR: $n = 8$; BR: $n = 1$) and samples contaminated with urine (WR: $n = 3$; BR: $n = 1$) were discarded and excluded from further analyses, including the statistical comparisons.

Descriptive statistics of the semen-rich fraction volumes and microscopic sperm variables are presented in Table I. Significant differences between EE and UC collected samples, were only found in semen volume ($p = 0.049$) and sperm concentration ($p = 0.036$). The data of the variables were combined within each

species irrespective of the method of collection as no significant differences were detected (Table I).

The effect of time between collection and evaluation was not evaluated statistically as only one BR and four WR samples could not be processed within 30 min of collection. However, motility and vitality were within the range of samples processed within 30 min (total motility: 40–70%, vitality: 81–87%).

Twenty-one (34%) WR bulls were housed with and 41 (66%) without access to females. Nine (43%) samples were collected from the bulls housed with females, of which two (10%) were good and seven (33%) were poor quality samples. In the group of bulls housed without access to females, 27 (66%) samples were collected, whereof 13 (32%) were good and 14 (34%) poor quality samples. The total sperm count and sperm with intact acrosomes (% intact) were significantly higher in the bulls housed without females ($p = 0.035$ and $p = 0.042$, respectively). No association between successful semen collection and social status was detected ($p = 0.22$).

Table I: Volume and sperm concentration, total sperm count, total and progressive motility, morphology, intact acrosomes and viability of 36 white (WR) and four black rhinoceros (BR) semen samples. Samples were categorised as “good” ($\geq 45\%$ for each of the following parameters: % motile sperm, % normal sperm, % intact and % viable sperm and $> 25 \times 10^6$ sperm/mL) or “poor” quality sample (one or more of the before mentioned parameter below 45%). Semen volume and sperm concentration differed significantly between samples collected via electro-ejaculation (EE) and urethral catheterisation (UC) in WR and are presented separately. For all other parameters, the findings of the two collection methods were combined. Semen-rich samples of 11 WR and 2 BR were excluded from analyses due to urine contamination or very low numbers of motile or only non-motile sperm. Values are provided as median (range) and mean $\pm$ SD to allow comparison with previous reports.

Parameter	White rhinoceros						Black rhinoceros (n = 4)	
	Good quality samples (n = 15)		Poor quality samples (n = 21)		Good and poor quality samples (n = 36)		Median (range)	Mean $\pm$ SD
	Median (range)	Mean $\pm$ SD	Median (range)	Mean $\pm$ SD	Median (range)	Mean $\pm$ SD		
Semen volume (ml)	12.3 (4–64)	20.4 $\pm$ 18.4	10.3 (1–57.5)	16.3 $\pm$ 16.5	EE: 14.5 (0.8–64) UC: 8.3 (3.0–11.0)	EE: 20.3 $\pm$ 17.4 UC: 7.4 $\pm$ 3.4	12.1 (5–18)	12.0 $\pm$ 3.6
Sperm Concentration (sperm $\times 10^6$ /ml)	69.0 (27–205)	94.9 $\pm$ 66.3	34.0 (9–93)	44.8 $\pm$ 31.2	EE: 45.0 (9.0–93.0) UC: 145.5 (30.0–205.0)	EE: 44.4 $\pm$ 28.2 UC: 131.5 $\pm$ 75.3	13.0 (5–54)	24.0 $\pm$ 26.7
Total sperm count (sperm $\times 10^6$)	1240.0 (551–3392)	1479.1 $\pm$ 974.3	222.0 (34–2557)	673.1 $\pm$ 934.1	684.1 (34–3392)	1025.7 $\pm$ 1007.8	208.0 (36–702)	315.3 $\pm$ 345.7
Total motility (% motile sperm)	60.0 (50–70)	59.2 $\pm$ 7.6	40.0 (10–70)	45.0 $\pm$ 15.1	50.0 (10–70)	51.0 $\pm$ 14.2	60.0 (30–70)	55.0 $\pm$ 17.3
Progressive motility (% progressively motile sperm)	40.0 (20–50)	40.0 $\pm$ 9.1	20.0 (5–50)	24.7 $\pm$ 11.4	30.0 (5–50)	31.1 $\pm$ 12.9	40.0 (20–50)	37.5 $\pm$ 12.6
Sperm morphology (% normal sperm)	62.0 (46–66)	59.1 $\pm$ 6.6	40.0 (5–68)	38.0 $\pm$ 17.8	44.0 (5–68)	44.0 $\pm$ 17.1	56.5 (49–80)	60.5 $\pm$ 14.5
Intact acrosomes (%)	89.0 (81–96)	88.4 $\pm$ 4.7	81.0 (32–95)	74.5 $\pm$ 16.9	85.0 (32–96)	80.0 $\pm$ 15.1	89.5 (77–96)	89.5 $\pm$ 8.5
Sperm viability (% viable sperm)	88.0 (81–97)	88.6 $\pm$ 4.6	81.0 (32–95)	74.3 $\pm$ 17.01	85.0 (32–97)	79.9 $\pm$ 15.0	90.0 (77–96)	88.3 $\pm$ 8.4

Table II: Comparison of semen volume, sperm concentration, total sperm count, total and progressive motility, morphology, intact acrosomes and sperm viability between samples obtained with electro-ejaculation (EE) and urethral catheterisation (UC) and between samples collected from bulls housed with vs. without females using a Mann Whitney U test. Significant differences are indicated (*).

	Mann Whitney U test	
	p-value EE vs. UC	p-value Bulls housed with vs. without females
Semen volume (ml)	0.049*	0.820
Sperm concentration (sperm $\times 10^6$ /ml)	0.036*	0.144
Total sperm count (sperm $\times 10^6$)	0.457	0.350*
Total motility (% motile sperm)	0.720	0.798
Progressive motility (% progressively motile sperm)	0.526	0.763
Sperm morphology (% normal sperm)	0.107	0.541
Intact acrosomes (%)	0.568	0.042*
Sperm viability (% viable sperm)	0.964	0.053

The most prevalent morphological abnormality was normally shaped detached heads and bent midpieces observed in 35/36 (97%) WR and 4/5 (80%) BR samples. The most common other defects were proximal (32/36; 89% and 3/5; 60%) and distal droplets (26/36; 72% and 4/5; 80%), and Dag-like defects (25/36; 69% and 1/5; 20%). Overall, WR samples showed 23 (5–86) primary and 26 (6–66) secondary abnormalities, and 5 (0–69), 25 (8–80) and 8.5 (0–41) head, midpiece and tail defects, respectively. BR samples presented with 18.0 (10.0–24.0) primary and 25.5 (10–32) secondary abnormalities, and 2 (0–4), 33.5 (12–54), 4 (2–12) head, midpiece and tail defects, respectively.

Discussion

Here, potential factors facilitating semen collection under field conditions in free-roaming African rhinoceroses were assessed. Despite time constraints and partly difficult climatic and terrain conditions, semen collection was successfully combined with management interventions in both species. This is the first report of semen collection in free-ranging BR.

The design of a species-specific electro-ejaculator probe allowed for optimal placement, facilitating collection. UC was successful following a few sets of electro-stimulation and shortened the

time required for semen collection. Overall, semen quality described in this study, despite considerable variation between the individuals, compared well to the quality reported in previous reports involving mainly captive animals. The experience of the operator appears to influence the success rate of collection.

Semen collection

EE probe

The custom-made probe enabled the efficient placement in both rhinoceros species, even with animals in sternal recumbency. The upward angle of the handle in relation to the probe differed from the downward-directed handle of a previously described probe (Roth et al. 2005). The downward handle would be difficult to position, as the ground would be in the way, especially if males are placed in sternal recumbency.

A maximum of 10 V and 277 mA was utilised in this study and overall resulted in good semen quality. While similar voltages were successfully deployed in greater one-horned rhinoceroses (Roth et al. 2005), much higher voltages were employed for semen collection in captive white rhinoceroses (20–29 V and 900 mA) (Hermes et al. 2005). Our results suggest that the custom-built probe employed in this study allowed for optimal placement and hence good contact between the electrodes and rectal mucosa adjacent to the target areas. As a result, lower voltages could be used which helps to reduce the incidence of urine contamination.

EE and UC techniques

Ultrasound examination proved valuable for optimal placement of the probe, especially during the initial phase. The ability to maintain the probe position, interpret responses such as leg movement, scrotal contractions and retraction of the penis, improved with experience, and allowed suitable adjustments to be made. Success was dependent on good communication between the person operating the electro-ejaculator, the probe handler, and the person responsible for holding the penis and exchanging the condom sleeves (semen collector).

As previously suggested (Roth et al. 2005), transrectal massage of the accessory glands before commencement and between each set of stimulations, appeared to facilitate semen emission. The initial massage also assisted in relaxation and manual extrusion of the penis. The BR in this study reacted particularly well to massaging and sperm-free fractions were recovered from two animals before electro-stimulation had commenced. Massaging the perineal region and thus the penile urethra from the anus towards the scrotum between EE stimulation sets appeared to facilitate semen flow. Contrary to other reports in rhinoceroses (Roth et al. 2005; Walzer et al. 2000), penile massage was not employed. Excessive handling of the penis such as increased traction to extrude the penis further, often resulted in penile retraction. Full erection during electro-stimulation was not always present and, in accordance with the findings of a previous study (Roth et al. 2005), was not a prerequisite for successful semen collection. Pulsatile ejaculation was only observed in a few rhinoceroses.

The average time required to obtain semen samples from rhinoceroses has only been reported in one study and, at 33.8 ± 2.0 (20–45) min, was considerably longer than reported in this study. To maximise the number of stimulations during the time available for semen collection, rest periods between sets, were shortened to approximately one min. Previously, rest periods of up to 5 min were applied (Roth et al. 2005).

Interestingly, the highest semen collection success rate in WR was achieved in the group immobilised with the higher doses of medetomidine. This finding is in accordance with previous findings in other species (Cavalero et al. 2019; Lueders et al. 2012; Zambelli et al. 2007). Semen collection attempts in WR immobilised with etorphine-azaperone combinations by the same team (not included in this study), although successful, only resulted in dilute, low volume samples. Specific α_2 -adrenoceptor agonists, such as medetomidine, have been associated with increased rates of semen emission and ejaculation in domestic animals, as both processes are α -adrenergically mediated (Zambelli et al. 2007).

In-the-field collections were associated with several challenges, including terrain and weather conditions. Covering the tip of the penis with the modified rectal sleeve, which acted as a funnel for the insulated collection tube, reduced the risk of contaminating the semen sample with dirt and exposing it to temperature shock. Changing the semen capture sleeve after each stimulation set was also a precaution to avoid urine contamination of the sample. Once urination was observed, semen collection was aborted as good quality semen samples following that, were no longer expected. No specific pattern or cause of urine contamination was identified previously (Roth et al., 2005). In this study, urine contamination appeared to be associated with a more cranial positioning of the probe leading to stimulation of the bladder neck. Therefore, the short length of the electrodes paired with probe placement as far caudal as possible, need special attention when collecting semen in African rhinoceroses.

In several wild felid and canid species, immobilised with ketamine and medetomidine, UC has been utilised as an alternative method for semen collection (Franklin et al. 2018; Lueders et al. 2012). In one black rhinoceros immobilised with etorphine-ketamine-azaperone, UC was employed as the sole method of semen collection, and small volume samples of good quality could be recovered (Moresco et al. 2015). In the later stages of the current study, UC was employed in rhinoceroses following the development of an erection and/or contractions of the scrotum in response to electro-stimulation. As semen is released by the vasa differentia (emission) and the accessory sex glands into the urethra prior to ejaculation, the time taken to obtain samples could be reduced to less than 15 minutes using UC. This is a major advantage when working with wildlife. In addition, UC allowed recovery of samples that would otherwise have been lost if there was a need to reverse anaesthesia. Small fractions of semen appear to be present in the urethra and UC may be the only option to recover these. The combination of a low number of electro-stimulation sets followed by UC appears to be promising as an alternative method for semen collection in wildlife where limited time is available and should be further investigated.

Smaller diameter (3.3 mm vs. 6.6 mm) tubes, were preferred to create adequate capillary flow. A recent study comparing UC to EE described the use of UC without prior EE stimulation (Moresco et al. 2024). The immobilisation drug combination used in our study was insufficient to provoke spontaneous semen emission and, unfortunately, doses used in the study mentioned above were not provided. Their UC method differed in that a smaller size catheter (5–7 FG) was inserted up to 110 cm and a very low negative pressure was applied (3 ml syringe with 0.5 ml suction). This may be important to avoid aspiration of the urethral mucosa. Semen volumes obtained were similar to this study.

Semen quality

Similar to other studies, ejaculate volume and microscopic sperm variables between individuals in both rhinoceros species differed considerably (Hermes et al. 2005; Reid et al. 2009; Roth et al. 2005).

Large volumes of seminal plasma were obtained from the first 32 WR sampled. This was possibly due to a more cranial positioning of the probe at the beginning of the study, which would increase stimulation of the seminal vesicles. In the interests of animal welfare, semen collection was terminated as soon as a potential semen-rich sample had been recovered. This possibly explains the lower mean ejaculate volumes observed compared to previous reports in white rhinoceroses (Hermes et al. 2005; 2018), where stimulation continued until clear seminal fluid was observed. Volumes obtained with UC were significantly lower and similar to UC collections in captive African rhinoceroses (Moresco et al. 2024).

Sperm concentration was within the range (Hermes et al. 2005) or slightly lower (Reid et al. 2009) than observed in previous reports in WR and at least 10-fold lower than reported for the greater one-horned rhinoceroses (Roth et al. 2005). This may be due to species differences, as epididymal sperm collection also resulted in significantly higher total sperm counts in greater one-horned rhinoceroses than in either African rhinoceros species (Roth et al. 2016).

In WR, the environmental conditions, transport and time taken from collection site to evaluation of samples in the field laboratory may partly explain the lower total motility observed in this study, as opposed to observations in captive animals (Hermes et al. 2005; Moresco et al. 2024; Reid et al. 2009; Roth et al. 2005). Compared to previous observations in rhinoceroses, the percentage morphologically normal sperm, viable sperm and intact acrosomes were similar (Hermes et al. 2005; Hildebrandt et al. 2018; Luther 2016; Moresco et al. 2024; Roth et al. 2016) and slightly higher in BR than in WR (Table I). Interestingly, total sperm count and the % intact acrosomes were higher in males without access to females. The sperm count can potentially be explained by regular mating in the males housed with females. However, we have no explanation for the observed increase of loose acrosomes as we would have expected the opposite as a result of sperm aging due to non-voidance in the distal parts of the vasa differentia when ejaculation occurs less regularly.

The most prevalent morphological abnormalities (normal detached heads, bent mid- or principal pieces, proximal and

distal droplets, and Dag-like defects) conform with previous reports in rhinoceroses (Hermes et al. 2005; Lubbe et al. 1999; Roth et al. 2005). With the exception of Dag-like defects, these abnormalities are often associated with sperm maturation/aging in the epididymis and ampullae (Nöthling & Irons 2008), but may also be artefacts resulting from mechanical injury, cold and hypotonic shock (Barth & Oko 1989).

Both techniques of semen collection allowed for the collection of good quality samples. In a recent study (Moresco et al. 2024), no difference in sperm post-thaw quality parameters were found between samples collected with either EE or UC. This highlights the potential for UC collection of semen during time-sensitive events. However, the technique needs further investigation.

Study limitations

Semen collection depended on opportunistic sampling events of rhinoceroses and the time available for semen collection depended on the environmental conditions. Although attempted here, procedures, could not be truly standardised and findings are largely descriptive. Collections were carried out by the same team, yet confounding factors (probe placement, experience levels, time to evaluation, outside temperature, environmental and social factors) may have influenced findings and were not considered in the statistical analysis. Results therefore should be interpreted with caution. Also, previously published reports, where mostly captive animals were included, may only be compared to the current study to a limited extent.

Nevertheless, inexpensive techniques and equipment applied in this study enabled the collection of semen-rich fractions in wild individuals, despite time constraints and varying environmental conditions and are encouraging the implementation of semen collection, analysis and cryopreservation into any male rhinoceros' anaesthesia.

Conclusions

This is the first report of routine management interventions such as dehorning or ear-notching that require immobilisation of free-ranging and game-farmed African rhinoceroses, being successfully utilised for semen collection. Although semen quality varied considerably, it was possible to collect good quality samples using the techniques and equipment described, despite the challenges of limited available time and environmental conditions. Semen samples suitable for cryopreservation may be used to establish genetic biobanks for these two species. Factors, which are likely to influence the semen quality are operator experience, probe design, immobilisation protocol, and collection technique. Additional modifications of these protocols may improve semen collection success rates, as well as semen quality. UC following rectal massage or pre-collection electro-stimulation, promises to be a useful method of semen collection in rhinoceroses, especially as the time required for the procedure can be reduced considerably.

Conflict of interest

The authors declare they have no conflicts of interest that are directly or indirectly related to the research.

Funding sources

This work was entirely funded by Hemmersbach Rhino Force SA NPC.

Ethical approval

Prior to the commencement of the study, ethical approval was obtained from the following ethical review board: University of Pretoria Animal Ethics and Research Committee (REC113-19).

ORCID

J Meuffels-Barkas  <https://orcid.org/0000-0001-8459-0834>

I Luther-Binoir  <https://orcid.org/0000-0003-0969-6895>

H Bertschinger  <https://orcid.org/0000-0001-8949-1990>

I Callealta  <https://orcid.org/0000-0002-7028-4139>

B Tindall  <https://orcid.org/0000-0001-8007-2167>

I Lueders  <https://orcid.org/0000-0003-3508-0066>

References

- Arnold, D.M., Gray, C., Roth, T.L., et al., 2017, A simple, field-friendly technique for cryopreserving semen from Asian elephants (*Elephas maximus*), *Anim Reprod Sci* 182, 84–4. <https://doi.org/10.1016/j.anireprosci.2017.05.003>.
- Ballou, J.D., Lacy, R.C., Traylor-Holzer, K., et al., 2023, Strategies for establishing and using genome resource banks to protect genetic diversity in conservation breeding programs, *Zoo Biol* 42, 175–84. <https://doi.org/10.1002/zoo.21741>.
- Barth, A.D., Oko, R., 1989, Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames, Iowa. Available from: <https://www.scirp.org/reference/referencespapers?referenceid=2435418>.
- Cavaleiro, T.M.S., Papa, F.O., Schmith, R.A., et al., 2019, Protocols using detomidine and oxytocin induce ex copula ejaculation in stallions, *Theriogenology* 140, 93–8. <https://doi.org/10.1016/j.theriogenology.2019.08.024>.
- Emslie, R., 2020a, *Ceratotherium simum*. IUCN Red List Threat, *Species* 2020 e.T4185A45.
- Emslie, R., 2020b, *Diceros bicornis*. IUCN Red List Threat, *Species* 2020 e.T6557A15. <https://doi.org/10.2305/IUCN.UK.2020-1.RLTS.T6557A152728945.en>.
- Franklin, A.D., Waddell, W.T., Goodrowe, K.L., 2018, Red wolf (*Canis rufus*) sperm quality and quantity is affected by semen collection method, extender components, and post-thaw holding temperature, *Theriogenology* 116, 41–8. <https://doi.org/10.1016/j.theriogenology.2018.05.007>.
- Hermes, R., Hildebrandt, T.B., Blottner, S., et al., 2005, Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum*, *C.s. cottoni*): Evaluation of male genital tract morphology and semen quality before and after cryopreservation, *Theriogenology* 63, 219–238. <https://doi.org/10.1016/j.theriogenology.2004.04.007>.
- Hermes, R., Hildebrandt, T.B., Göritz, F., 2018, Cryopreservation in rhinoceros—setting a new benchmark for sperm cryosurvival, *PLoS One* 13, 1–12. <https://doi.org/10.1371/journal.pone.0200154>.
- Hildebrandt, T.B., Hermes, R., Colleoni, S., et al., 2018, Embryos and embryonic stem cells from the white rhinoceros, *Nat Commun* 9. <https://doi.org/10.1038/s41467-018-04959-2>.
- Jeong, D.H., Kim, J.H., Na, K.J., 2018, Characterization and cryopreservation of Amur leopard cats (*Prionailurus bengalensis euptilurus*) semen collected by urethral catheterization, *Theriogenology* 119, 91–5. <https://doi.org/10.1016/j.theriogenology.2018.06.004>.
- Lubbe, K., Smith, R., Bartels, P., et al., 1999, Freezing epididymal sperm from white rhinoceroses (*Ceratotherium simum*) treated with different cryodiluents, *Theriogenology* 51, 288. [https://doi.org/10.1016/S0093-691X\(99\)91847-2](https://doi.org/10.1016/S0093-691X(99)91847-2).
- Lueders, I., Luther, I., Scheepers, G., et al., 2012, Improved semen collection method for wild felids: Urethral catheterization yields high sperm quality in African lions, *Theriogenology* 78, 696–701. <https://doi.org/10.1016/j.theriogenology.2012.02.026>.
- Luther, I., 2016, Semen characteristics of free-ranging African elephants (*Loxodonta africana*) and Southern white rhinoceros (*Ceratotherium simum simum*) using Computer-aided sperm analysis, Electron microscopy and Genomics as diagnostic tools. University of the Western Cape, Bellville.
- McGowan, M., 2018, Evaluation of the fertility of breeding males, Tenth Edit. ed, Veterinary Reproduction & Obstetrics. Elsevier Ltd. <https://doi.org/10.1016/B978-0-7020-7233-8.00035-5>.
- Meuffels, J., Bertschinger, H., Tindall, B., et al., 2022, Arterial blood gases and cardiorespiratory parameters in etorphine-medetomidine-midazolam immobilized free-ranging and game-farmed Southern White Rhinoceroses (*Ceratotherium simum simum*) undergoing electro-ejaculation, *Front Vet Sci Zool Med* 9. <https://doi.org/10.3389/fvets.2022.862100>.
- Meuffels, J., Lueders, I., Bertschinger, H.J., et al., 2021, Cardiopulmonary parameters and arterial blood gases during etorphine-medetomidine-midazolam immobilization in free-ranging black rhinoceroses (*Diceros bicornis*) undergoing electro-ejaculation - a preliminary study, *Front Vet Sci Zool Med* 8. <https://doi.org/10.3389/fvets.2021.740614>.
- Milligan, S., Holt, W., Lloyd, R., 2009, Impact on climate change and environmental factors on reproduction and development in wildlife, *Phil Trans R Soc B* 364, 3313–9. <https://doi.org/10.1098/rstb.2009.0175>.
- Moresco, A., Larsen, R.S., Boon, D., et al., 2015, Semen collection in Black Rhinoceros (*Diceros bicornis*) via urethral catheterization, *Proc Annu Conf AAZV* 170–1.
- Moresco, A., O'Brien, J.K., Wojtusik, J., et al., 2024, Sperm collection in rhinoceros via urethral catheterization, *Theriogenology Wild* 4, 100090. <https://doi.org/10.1016/j.therwi.2024.100090>.
- Morkel, P.vdB., Radcliffe, R.W., Jago, M., et al., 2010, Acid-base balance and ventilation during sternal and lateral recumbency in field immobilized black rhinoceros (*Diceros bicornis*) receiving oxygen insufflation: A preliminary report, *J Wildl Dis* 46, 236–45. <https://doi.org/10.7589/0090-3558-46.1.236>.
- Nhleko, Z.N., Ahrens, R., Ferreira, S.M., et al., 2021, Poaching is directly and indirectly driving the decline of South Africa's large population of white rhinos, *Anim Conserv* 1–13. <https://doi.org/10.1111/acv.12720>.
- Nöthling, J.O., Irons, P.C., 2008, A simple multidimensional system for the recording and interpretation of sperm morphology in bulls, *Theriogenology* 69, 603–11. <https://doi.org/10.1016/j.theriogenology.2007.11.007>.
- Prieto, M.T., Hildebrandt, T.B., Saragusty, J., 2014, Sperm cryopreservation in wild animals, *Eur J Wildl Res* 60, 851–64. <https://doi.org/10.1007/s10344-014-0858-4>.
- R Development Core Team, 2019, R: A Language and Environment for Statistical Computing.
- Reid, C.E., Hermes, R., Blottner, S., et al., 2009, Split-sample comparison of directional and liquid nitrogen vapour freezing method on post-thaw semen quality in white rhinoceroses (*Ceratotherium simum simum* and *Ceratotherium simum cottoni*), *Theriogenology* 71, 275–91. <https://doi.org/10.1016/j.theriogenology.2008.07.009>.
- Roth, T.L., Stoops, M.A., Atkinson, et al., 2005, Semen collection in rhinoceroses (*Rhinoceros unicornis*, *Diceros bicornis*, *Ceratotherium simum*) by electroejaculation with a uniquely designed probe, *J Zoo Wildl Med* 36, 617–27. <https://doi.org/10.1638/05-019.1>.
- Roth, T.L., Stoops, M.A., Robeck, T.R., et al., 2016, Factors impacting the success of post-mortem sperm rescue in the rhinoceros, *Anim Reprod Sci* 167, 22–30. <https://doi.org/10.1016/j.anireprosci.2016.01.019>.
- Walzer, C., Pucher, H., Schwarzenberger, F., 2000, A restraint chute for semen collection in white rhinoceros (*Ceratotherium simum simum*) -preliminary results, in: European Association of Zoo and Wildlife Veterinarians. Paris, pp. 7–10.
- Zambelli, D., Cunto, M., Prati, F., et al., 2007, Effects of ketamine or medetomidine administration on quality of electroejaculated sperm and on sperm flow in the domestic cat, *Theriogenology* 68, 796–803. <https://doi.org/10.1016/j.theriogenology.2007.06.008>.