Journal of Zoo and Wildlife Medicine 36(4): 617–627, 2005

SEMEN COLLECTION IN RHINOCEROSES (*RHINOCEROS UNICORNIS*, *DICEROS BICORNIS*, *CERATOTHERIUM SIMUM*) BY ELECTROEJACULATION WITH A UNIQUELY DESIGNED PROBE

Terri L. Roth, M.S., Ph.D., Monica A. Stoops, M.S., Ph.D., Mark W. Atkinson, B.V.Sc., M.R.C.V.S, Evan S. Blumer, M.S., V.M.D., Mark K. Campbell, D.V.M., Ken N. Cameron, D.V.M., Scott B. Citino, D.V.M., Dipl. A.C.Z.M., and Adolf K. Maas, D.V.M.

Abstract: Electroejaculation in rhinoceroses has historically yielded inconsistent results, with the collection of high-quality, sperm-rich samples rare. The goal of this study was to develop a reliable method of electroejaculation in the rhinoceros by designing a rectal probe that appropriately fits the anatomy of this taxon and refining the procedure. A curved probe handle ending in an oblate, ellipsoid head was built using readily available supplies. A combination of rectal massage, penile massage, and electrical stimulation with a specially designed probe was employed in attempts to collect semen on 14 occasions from greater one-horned rhinoceroses (*Rhinoceros unicornis*; $n = 4$), black rhinoceroses (*Diceros bicornis*; $n = 2$) and a southern white rhinoceros (*Ceratotherium simum*; $n = 1$). During 13 of the 14 attempts, ejaculates were collected in multiple fractions. All but one of the ejaculates contained spermatozoa, and seven ejaculates contained good-quality fractions of semen ($\geq$60% sperm motility; $\geq 20 \times 10^6$ spermatozoa/ml) suitable for sperm banking and assisted reproduction procedures. Mean ($\pm$ SEM) values for volume, pH, osmolality, and total sperm number for ejaculates containing good-quality fractions (98.2 $\pm$ 21.8 ml, 8.5 $\pm$ 0.1, 290.4 $\pm$ 6.7 mOsm, and 37.1 $\pm$ 12.0 $\times 10^9$, respectively) did not differ ($P > 0.05$) from those containing only poor-quality samples. Urine and/or erythrocyte contamination was not uncommon in fractions of both ejaculate types. Males producing good-quality samples ranged in age from 7 to 34 yr. None of the samples contained $\geq$75% morphologically normal spermatozoa. Electroejaculation with a uniquely designed probe consistently produced ejaculates in the rhinoceros. However, the production of high-quality samples continued to be challenging, occurring in only 50% of collection attempts. Regardless, the technology has progressed to a stage at which good-quality semen samples can be produced for sperm banking and assisted reproduction, and thereby can be integrated into intensive rhinoceros management strategies for the ultimate survival of this taxon.

Key words: Assisted reproduction, black rhinoceros, *Diceros bicornis*, greater one-horned rhinoceros, *Rhinoceros unicornis*, spermatozoa, white rhinoceros, *Ceratotherium simum*.

INTRODUCTION

Semen collection has become standard practice in many domestic animal breeding programs for fertility evaluations and assisted reproduction procedures. As the management of endangered species improves and intensifies, the ability to collect semen from these nondomestic animals is becoming increasingly important for the same reasons it has become an integral component of breeding programs for their domestic counterparts. Furthermore, with limited extant genetic diversity, endangered populations may be forced to rely on gene banks for infusions of diverse genetic material in the future. Therefore, there is an urgent need to collect and cryopreserve spermatozoa from individuals representing the current gene pool of small populations.

The rhinocerotidae is one taxon that could significantly benefit from a reliable method of semen collection. Four of the five rhinoceros species (black rhinoceros, *Diceros bicornis*; greater one-horned rhinoceros, *Rhinoceros unicornis*; Sumatran rhinoceros, *Dicerorhinus sumatrensis*; and Javan rhinoceros, *Rhinoceros sondaicus*) are endangered, with population estimates currently as low as 60 animals (Javan rhinoceros) and no higher than 3,610 (black rhinoceros; www.rhinos-irf.org). Therefore, preserving spermatozoa from representatives of the current population is an important component of a broader strategy to prevent further loss of genetic variation. Additionally, research is underway to develop artificial insemination (AI) in rhinoceroses to assist captive breeding efforts in producing offspring from genetically matched animals that are behaviorally incompatible.[7,23] Semen collection and sperm banking are integral to the de-

From the Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, 3400 Vine Street, Cincinnati, Ohio 45220, USA (Roth, Stoops, Campbell, Cameron); The Wilds, 14000 International Rd., Cumberland, Ohio 43732, USA (Atkinson, Blumer); White Oak Conservation Center, 3823 Owens Rd., Yulee, Florida 32097, USA (Citino); and Exotic and Companion Animal Veterinary Services, 4621 E. Viola Dr., Mooresville, Indiana 46198, USA (Maas). Present address (Atkinson): Montana Fish, Wildlife and Parks, 1400 S. 19th St., Bozeman, Montana 59718, USA. Correspondence should be directed to Dr. Roth.

velopment and application of this technology. Furthermore, the fertility of specific individual male rhinoceroses has been questioned because frequent, confirmed matings by these males have been observed but no pregnancies have been confirmed.[18]

Semen collection by artificial vagina (AV) has been largely unsuccessful in the rhinoceros.[21] Penile massage has resulted in sperm production on a few occasions, but the success of this procedure depends largely on the animal's temperament, can require years of training and effort, and only rarely produces high-quality samples.[21] Postcoital semen collection from a female Sumatran rhinoceros yielded high-volume, good-quality samples,[13] but this semen collection strategy will not be broadly applicable and cannot be considered for male rhinoceroses that are not mating with females. Electroejaculation, the most commonly used method for collecting semen from nondomestic species, has been used with some success in rhinoceroses in the past.[15,20] However, the technique is challenging in this taxon and historically has been unreliable in producing high quality samples that can be used for cryobanking or assisted reproduction.[15,20] However, a recent report indicates that electroejaculation using a specially designed, handheld, rectal probe improves the effectiveness for semen collection in the white rhinoceros.[5]

Failure of electroejaculation to produce good-quality semen samples in the rhinoceros could be attributed to improper and insufficient stimulation of the reproductive glands resulting from a rectal probe that does not properly fit the rhinoceros rectal anatomy. Standard rectal probes typically are linear with longitudinal electrodes. However, the rhinoceros reproductive glands are adjacent to the neck of the bladder and lie ventrally in the caudalmost portion of the rectum just inside and below the anal sphincter.[20,22] Therefore, a linear rectal probe angled down inside the anal sphincter is more effective for making contact with the rectal lining cranial to the glands.

This study was designed to test the hypothesis that a custom-made, externally-held rectal probe would improve the success of electroejaculation and provide a more consistent and reliable method for semen collection in the rhinoceros.

MATERIALS AND METHODS

Animals

Seven rhinoceroses were included in this study: four greater one-horned rhinoceroses and three African rhinoceroses (one eastern black [*Diceros bicornis michaeli*], one southern black [*Diceros bicornis minor*] and one southern white [*Ceratotherium simum*]). Most procedures were performed either opportunistically in conjunction with other medical procedures or in an effort to answer questions concerning an animal's reproductive fitness.

During the early phases of this study, a single male greater one-horned rhinoceros (No. 87) was used. This animal was maintained at The Wilds in Cumberland, Ohio, and had arrived there with serious foot problems that had to be treated on a regular basis. Therefore, the electroejaculation procedures were conducted opportunistically when the male was anesthetized for foot treatments, and a relatively high number of procedures ($n = 6$) were conducted on this animal. He was 22–26 yr old during the study. A second male also maintained at The Wilds was the youngest animal in the study at just 7 yr of age (No. 239). He was being placed in a breeding situation with two female rhinoceroses and his pubertal status was questioned. Electroejaculation was performed, in part, to determine whether he had attained puberty and was producing spermatozoa.

Male greater one-horned rhinoceros No. 147 was maintained at the Cincinnati Zoo and Botanical Garden. Although a relatively young animal during this study (13–15 yr), he had developed severe lameness and significant muscle atrophy associated with undefined pathology in the left coxofemoral joint. He was deemed unfit for natural breeding, and electroejaculation was performed three times to collect semen for sperm cryobanking and for use in AI procedures. A second male rhinoceros at the Cincinnati Zoo included in the study was an Eastern black rhinoceros (No. 247) that was wild-caught and estimated to be about 34 yr old at the time of electroejaculation. This male was a proven breeder that was suffering from severe arthritis in his shoulder and had to be euthanized. Electroejaculation was performed just before euthanasia. Spermatozoa were collected from the epididymides immediately following euthanasia to compare the quality of ejaculated samples to epididymal samples.

The final three rhinoceroses were maintained at White Oak Conservation Center in Yulee, Florida. Greater one-horned rhinoceros No. 49 was a wild-caught male estimated to be about 34 yr old. He was an unproven male that was housed with a reproductively cycling female with which he was never seen mating. Electroejaculation was performed, in part, to help rule out a possible physical impairment that could be contributing to his failed reproductive success. An unproven 33-yr-old male southern white rhinoceros (No. 469) had been

housed with a herd of female white rhinoceroses that were proven breeders at White Oak Conservation Center and was observed mounting females frequently, but none appeared to conceive. Therefore, electroejaculation was performed in an effort to confirm that the male was producing spermatozoa. Similarly, a young (17 yr) southern black rhinoceros (No. 523) had been housed with a female for many months, and the female did not appear to be conceiving. Although the male was a proven breeder, he had recently (within the year) recovered from a bout of hypervitaminosis D that led to the death of three other black rhinoceroses, and the return of his reproductive fitness was in question.

Rectal probe design and construction

The rhinoceros rectal probe for electroejaculation was designed with the goal of ensuring that the technician could make and maintain contact between the electrodes on the probe head and the accessory glands of the male rhinoceros located on the ventral aspect of the pelvic canal against the ischium.[22]

The probe consisted of an oblate, ellipsoid head containing three electrodes. The head of the probe was shaped in a manner that facilitated both insertion through the tight anal sphincter and electrode contact with the rectal lining over the accessory glands during stimulation. The probe assembly was constructed from a solid rod of polyvinyl chloride (PVC) stock, standard PVC pipe, flexible copper tubing, standard multistrand insulated copper wire, two-part epoxy putty, and appropriate adhesives. Figure 1a shows the complete probe assembly.

The probe head was formed from a solid rod of PVC stock cut as shown in Figure 1b (units are inches). A hole was drilled for the handle (Fig. 1c). The head was shaped and sanded using standard shop tools to round the outer edges (Fig. 1d). Shallow grooves were cut to accommodate the electrodes (Fig. 1e). At both ends of each groove, holes were drilled to fasten the ends of the electrodes. A large high-speed cutter bit (Model 194, Dremel Corporation, Racine, Wisconsin 53406, USA) was used to cut the grooves and drill the holes to attach the electrode ends. Smaller holes were drilled between the tip end of the electrode grooves and the handle hole to accommodate wires connecting to the electrodes.

The three electrodes were formed from sections of flexible ¼-inch copper tubing (Fig. 1f). At one end, the last ½ inch was crushed flat using a standard bench vise. The inside surface of the other end was scarified and treated with acid flux. The stripped end of a length of 16-ga stranded copper

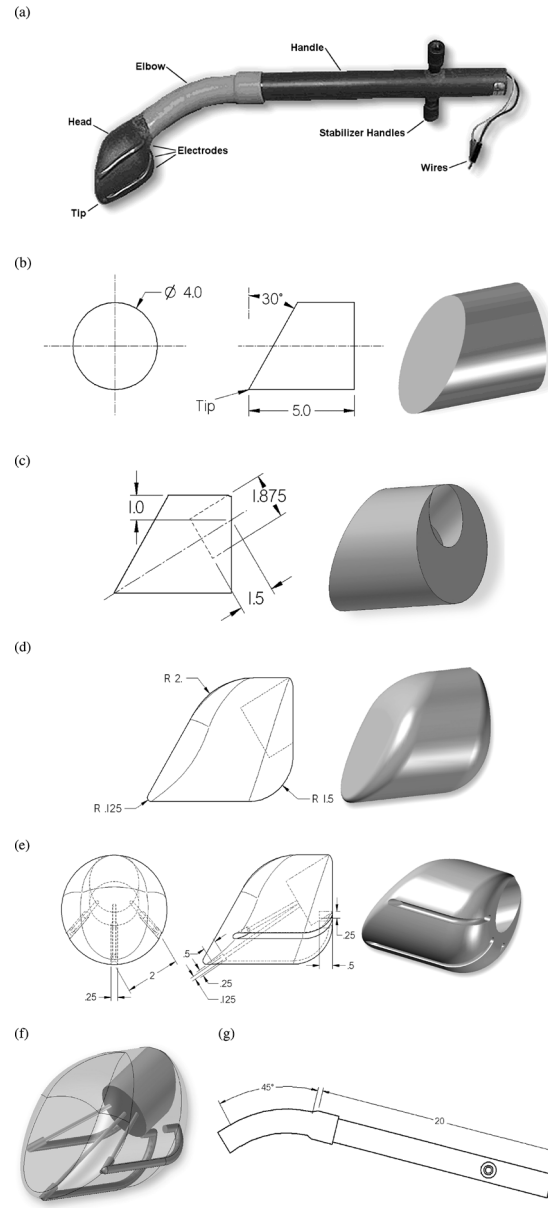

Figure 1. **a.** Computer modeling (Solid Edge, 3D modeling software, UGS Corporation, Plano, TX 750243, USA) images of the rhinoceros rectal probe construction resulting in the completed probe assembly. **b.** All measurements are in inches. PVC stock was cut to form the probe head. **c.** A hole for the handle was drilled in the probe head. **d.** The probe head was further shaped. **e.** Grooves were cut in the probe head to accommodate the electrodes. **f.** Wires extending from the handle hole were soldered to the electrodes. **g.** The handle was formed from two pieces of PVC pipe.

wire was inserted and subsequently crushed in the same manner as the first, but then soldered closed using a lead-free silver solder, to permanently affix the wire. Two-part epoxy putty (William H. Harvey Company, Omaha, Nebraska 68117, USA) was used to fill the holes and adhere the wired ends of the electrodes to the head. Once hardened, the electrodes were bent around the curve of the butt end, and their flattened ends were inserted into their respective holes at the end of the groove. Epoxy putty was used to bed the length of each electrode in its groove and permanently attach the free end to the head.

Three wires emerged at the hole drilled for the handle. The two wires from the lateral electrodes were soldered together to a single wire that matched the length of the third wire from the central electrode. All soldered connections were covered in heat-shrink plastic to provide insulation and prevent corrosion. The wires were fed through the handle, described next. Electrical connectors were added to the free ends of the wires for compatibility with the electroejaculator.

The handle was formed from two pieces of standard 1½-inch inner diameter (i.d.) PVC pipe: a straight piece and a 45° elbow with an attached coupler. The 45° elbow was cut at the end of the curve and inserted into the handle hole in the head of the handle (Fig. 1g). The joint between the elbow and the head was filled with epoxy putty and feathered smooth. The elbow was glued in place using clear PVC cement (Oatey Corporation, Cleveland, Ohio 44115, USA). A 20-inch section of 1½-inch i.d. PVC pipe was glued to the coupler end of the elbow. Stabilizing handles were added to the shaft of the handle. The open end of the handle was filled with latex spray foam to eliminate dead air space, and a sealing plug was installed around the wires using epoxy putty. All surfaces of the head (except electrodes) and the elbow were painted with acetone to remove oil and small defects, and then coated with a thin layer of diluted PVC cement to seal the surface. Prior to each use, surfaces of each electrode required sanding to remove oxidation. Following each use, the properly sealed probe was immersed in a bath of disinfectant cleaner.

Anesthesia

All rhinoceroses were fasted for 24 hr prior to anesthesia for electroejaculation. Water was withdrawn the morning of the procedure. During the procedure, 100% oxygen was administered through a nasal cannula by a demand valve resuscitator (JD Medical Distribution Co., Phoenix, Arizona 85029, USA). Respiration, pulse rate and O_2 saturation were monitored throughout the procedure.

The African eastern black rhinoceros at the Cincinnati Zoo was immobilized using a cocktail of etorphine (M99, 10 mg/ml, 3.5 mg, Wildlife Pharmaceuticals, Fort Collins, Colorado 80524, USA) and xylazine (Rompun, 100 mg/ml, 100 mg, Lloyd Labs, Shenandoah, Iowa 51601, USA) administered i.m. by projectile dart (Tel-Inject USA, Saugus, California 91350, USA) into the neck musculature. Following the procedure, the animal was euthanized as scheduled because of a preexisting medical condition.

The African southern black rhinoceros at White Oak Conservation Center was immobilized with etorphine (M99, 1.8 mg) and medetomidine hydrochloride (20 mg/ml, 3.0 mg, Wildlife Pharmaceuticals) administered i.m. by projectile dart (Tel-Inject USA). The animal received 5% guaifenesin (Guaifenesin Injection, The Butler Company, Columbus, Ohio 43228, USA) by i.v. drip throughout the procedure. Anesthesia was reversed with naltrexone (200 mg, 50 mg/ml i.v., Wildlife Pharmaceuticals) and atipamezole hydrochloride (Antisedan, 30 mg i.v., Pfizer Animal Health, Exton, Pennsylvania 19341, USA)

The African southern white rhinoceros was immobilized with butorphanol tartrate (Torphaject, 120 mg i.m., Vetus Animal Health, Burns Veterinary Supply, Inc., Westbury, New York 11590, USA) and medetomidine hydrochloride (5 mg i.m., Wildlife Pharmaceuticals) by projectile dart (Tel-Inject USA). During the procedure he was supplemented with ketamine (Ketaset, 100 mg/ml, 300 mg i.v., Fort Dodge Laboratories) and a 5% guaifenesin (Guaifenesin Injection, The Butler Company) by i.v. drip. Anesthesia was reversed with naltrexone (200 mg i.v., Wildlife Pharmaceuticals) and atipamezole hydrochloride (Antisedan, 30 mg i.v., Pfizer Animal Health).

All greater one-horned rhinoceroses at the three facilities were immobilized using a combination of etorphine (M99, 3.5–3.8 mg, Wildlife Pharmaceuticals), detomidine (Dormosedan, 10 mg/ml, 14–20 mg, Pfizer), and ketamine (200–400 mg, Fort Dodge Laboratories, Inc.) administered as a cocktail i.m. by projectile dart (Tel-Inject USA) or pole syringe (Dan-Inject Jabstick, Dan-Inject of North America, Fort Collins, Colorado 80527, USA) into the neck musculature. Supplemental ketamine was administered i.v. as needed, and one animal received guaifenesin (5% i.v. drip, Guaifenesin Injection, The Butler Company) during the procedure. At the conclusion of the procedures, the etorphine effects were antagonized by administration of nal-

trexone (80–200 mg i.v., Wildlife Pharmaceuticals). No reversal agent was used to antagonize the effects of detomidine.

Electroejaculation

Once the rhinoceros was sedated safely under a surgical plane of anesthesia, ropes were placed around the rear legs and anchored securely around the bars of the enclosure. The ropes did not completely restrict movement, but did keep personnel safe from any extended or rapid motion. Feces were removed from the rectum manually using a well-lubricated palpation sleeve. The penis was unsheathed and cleaned with water and a towel. The head of the rectal probe was well lubricated and slowly inserted into the animal's rectum just past the anal sphincter (Fig. 2a). Once inserted, the handle of the probe was lifted, forcing the head of the probe down at a slightly caudal angle within the rectum such that the electrodes made contact with the rectal lining just over the reproductive glands.

Electrical stimulation was administered using an electroejaculator (P-T Electronics, 11241 SE 362nd, Boring, Oregon 97009, USA) in several series of increasing voltage (+1 V each increase) with rest intervals of up to 5 min between each series. Each series typically consisted of 5–15 stimulations at each of three voltages, for a total of about 30 stimulations per series. Three to five series of stimulations were expected for each procedure. Later in the project, the probe was routinely removed and manual rectal massage administered during the 5-min rest periods between series. Throughout the procedure, the tip of the penis was held in a specimen cup to collect the ejaculate, and penile massage was frequently administered both during stimulation and throughout the rest period. Once the male started producing fluid, the collection cups were exchanged frequently to avoid potential contamination of a good-quality fraction with one of poorer quality.

Detailed data were collected during the procedure that included voltage, number of stimulations, milliamperage, penile response, fluid production, color of fluid, and opacity of fluid. The position of the probe was changed frequently by shifting it slightly left, right, cranially, or caudally during the procedure in an effort to find the best placement for stimulating penile responsiveness and ejaculate production and for achieving expected milliamperage readings. Maintaining the probe in proper position with good rectal contact was often difficult, especially at higher voltages when the animal's muscles contracted rather significantly.

Although the procedure could be conducted with fewer people, it was best when five individuals were available. One person handled the probe, the second delivered the stimulation by controlling the electroejaculator, the third recorded the data, the fourth massaged the penis, and the fifth kept the tip of the penis in a collection cup. Additional hands were required to take cups with sample fractions and supply new cups and to hold the ropes around the rhinoceros's hind legs (Fig. 2b). Additionally, the veterinary staff was needed to anesthetize and monitor the rhinoceroses during and after the procedure.

Sample processing and evaluation

All ejaculate fractions were examined for the presence of spermatozoa, urine, and red blood cells (RBC). A sample was considered contaminated with urine or RBC if there was the slightest hue of yellow or pink to it, respectively. Typically, while fractions pink or red in color were being examined for spermatozoa, the presence of RBC could be confirmed in the sample. Samples containing spermatozoa were evaluated for percent sperm motility and forward progression (scale of 0–5; 0 = no movement, 5 = rapid forward movement). Sample aliquots (10 μl) were added to 90 μl water and sperm concentration was determined using a Makler counting chamber (TS Scientific, Perkasie, Pennsylvania 18944, USA). Good-quality, sperm-rich fractions were defined as those containing relatively high concentrations of spermatozoa ($>20 \times 10^6$/ml) of which ≥60% were motile. For ejaculates containing good-quality fractions, those fractions were combined and aliquots (~10 μl) were fixed in 0.3% glutaraldehyde for analysis of sperm morphology. For ejaculates with only poor-quality fractions, all samples containing spermatozoa were combined and an aliquot used to assess sperm morphology. The morphology of 100 spermatozoa was evaluated, with each sperm cell recorded only once for its most severe abnormality. Ejaculate fractions were evaluated for pH by assessing the color change after adding 5 μl of sample to an indicator strip (EM Science, Gibbstown, New Jersey 08027, USA) and osmolality using a vapor pressure osmometer (Wescor, Logan, Utah 84321, USA).

Statistical analysis

Numeric data for ejaculates producing good-quality fractions versus those producing only poor-quality fractions were compared using the computer statistical software package Statview (Statview 5.0.1 MacIntosh, SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513, USA). Descriptive statistics were calculated and data were ana-

(a)

(b)

Figure 2. **a.** After anesthetizing the rhinoceros and removing feces from the rectum, the rectal probe was well lubricated and carefully inserted just past the anal sphincter. **b.** The procedure required a number of people, including one handling the probe, one running the electroejaculator, and several massaging the penis, collecting the sample, and trading out collection cups. The hind legs were secured with ropes, and the veterinary staff monitored anesthesia throughout the procedure.

lyzed by ANOVA, with sample quality serving as the independent variable and each characteristic as a dependent variable. $P < 0.05$ was considered significant. To determine if there were differences between the two ejaculate types in the proportions of contaminated samples, chi-square analysis was conducted.

RESULTS

Anesthesia and animal recovery

The anesthetic regimen for every animal was effective in producing an appropriate plane of anesthesia for the procedure. None of the animals in the study required any emergency resuscitation or experienced any unusual ill effects during anesthesia. The procedure averaged 33.8 ± 2.0 min (range, 20–45 min) from first to last stimulation. Typically, males were standing <5 min after receiving the reversal drugs. No procedure-induced soreness was observed in the animals the following day, and there were no reports of rectal bleeding or the presence of blood in fecal samples, which might have indicated irritation to the rectal lining from either excessive electrical stimulation or rectal massage.

Sample collection

Samples were successfully collected during 13 of the 14 electroejaculation procedures in males ranging in age from 7–34 yr. The first attempted collection on greater one-horned rhinoceros No. 147 failed to produce a sample during the procedure, but a highly concentrated 0.5-ml sperm-rich sample was collected off the floor of the stall shortly after the male had recovered. Ten of the 13 samples collected were ≥70 ml in volume, and 12 of the 13 contained sperm-rich fractions. The only aspermic sample collected was from the 34-yr-old southern white rhinoceros (No. 469) that was of questionable fertility. Although 150 ml of seminal fluid was produced during the procedure, no sperm could be found in the sample. In most cases, ejaculates were collected in numerous fractions, with as many as 18 specimen cups used during a single procedure.

Urine and RBC contamination were not uncommon during the procedure. A total of eight collection attempts produced at least one contaminated ejaculate fraction. Four of these eight produced urine-contaminated fractions, two produced RBC-contaminated fractions, and two produced some fractions with urine and some with RBC contamination. Despite the contamination of some fractions, five of these eight attempts also produced good-quality ejaculate fractions.

Although all but one collection attempt produced sperm-rich fractions, the sperm quality in these fractions varied substantially, and good-quality fractions (those deemed appropriate for cryopreservation and/or artificial insemination) were sometimes difficult to obtain. A good-quality ejaculate fraction ($>20 \times 10^6$ spermatozoa/ml; ≥60% motile) was collected during seven of the procedures from the four greater one-horned rhinoceroses in the study. The male used most frequently (No. 87) produced good-quality fractions only 50% of the time, failing to produce a good-quality fraction during three of his six procedures. During each of these three failed attempts, a large-volume sample (>90 ml) containing spermatozoa was produced, but sperm motility was <10%. One other male greater one-horned rhinoceros (No. 147) who was subjected to three collection attempts also failed to produce a good-quality fraction during one of the collection attempts. The 0.5-ml sperm-rich fraction that was collected off the stall floor shortly after the procedure was highly concentrated with sperm cells, but motility was only ~3%. Whether the poor motility was the result of exposure to the elements for an extended period or whether sperm motility was poor when first produced is unknown. The other two male greater one-horned rhinoceroses (No. 49 and No. 239) both produced a good-quality fraction during their one collection attempt. Although the eastern black rhinoceros produced a concentrated sample (180×10^6 spermatozoa/ml), only 30% of its spermatozoa were motile. In contrast, the southern black rhinoceros produced spermatozoa with good motility (60%), but the samples were very dilute (3×10^6 spermatozoa/ml). Therefore, neither male contributed a good-quality sample to the study.

Procedures yielding good- versus poor-quality ejaculate fractions

Electroejaculation procedures did not differ significantly between attempts that yielded good-quality fractions and those that did not (Table 1). The maximum voltage used during the procedure and the total number of stimuli were similar ($P > 0.05$). The maximum milliamperage achieved was actually higher ($P < 0.05$) for attempts that did not yield quality samples. Although there was considerable variation among animals and attempts, ejaculation of seminal fluid typically occurred when the milliamperage reached 200–300 mA (usually 7–8 V) with concentrated, sperm-rich samples more likely to be produced at 300–400 mA.

The sample itself did not differ between the two groups in volume, pH, osmolality, or total spermatozoa produced ($P > 0.05$). The percentage of

Table 1. Electroejaculation parameters for procedures resulting in ejaculates with good-quality (n = 7) and poor-quality (n = 7) fractions and the characteristics of those fractions.[a]

Parameter	Ejaculates containing	
	Good quality fractions	Only poor quality fractions
Age of rhino (yr)	20.1 ± 3.3 (7–34)	24.1 ± 2.9 (13–34)
Maximum stimulation (V)	8.7 ± 0.4 (8–10)	9.7 ± 0.5 (8–12)
Maximum mA	315.7 ± 18.5[b] (250–400)	398.6 ± 29.3[c] (300–550)
No. stimuli	118.9 ± 18.6 (83–225)	126.9 ± 7.4 (98–150)
Ejaculate volume (ml)	98.2 ± 21.8 (36–200)	84.9 ± 21.6 (0.5–150)
Quality fraction volume (ml)	24.1 ± 4.0 (15–40)	NA
pH	8.5 ± 0.1 (7.9–9.0)	8.5 ± 0.2 (7.9–9.5)
Osmolality (mOsm/kg)	290.4 ± 6.7 (272–322)	299.2 ± 15.4 (262–368)
Total sperm ($\times 10^9$/ml)	37.1 ± 12.0 (1.8–66.4)	10.5 ± 6.4 (0–43)
Sperm motility (%)	80.7 ± 4.7[b] (60–90)	12.3 ± 7.5[c] (0–50)
Sperm progressive motility	2.9 ± 0.2[b] (2.0–3.5)	0.9 ± 0.5[c] (0–3.0)
Structurally normal sperm (%)	41.6 ± 6.1[b] (28–74)	21.0 ± 5.3[c] (4–37)
Urine contamination[d]	5	2
RBC contamination[d]	2	2

[a] Values are means ± SEM followed by ranges. RBC, red blood cell.

[b,c] Values with different superscripts within rows differ ($P < 0.05$).

[d] If any of the fractions contained urine or RBC during the electroejaculation procedure, the sample was counted as contaminated. The good-quality fraction itself did not contain urine or RBC.

structurally normal spermatozoa in the ejaculates was low (<75%) for all samples and likely contributed to the relatively low progressive motility ratings. Not surprisingly, samples containing good-quality fractions contained more morphologically normal spermatozoa than those producing only poor-quality fractions ($P < 0.05$). Urine and RBC contamination occurred during some collection attempts in both groups and were not more prevalent during attempts that produced only poor-quality fractions ($P > 0.05$).

DISCUSSION

The development of a uniquely designed, customized rectal probe that appropriately fits the rhinoceros anatomy was key in developing an electroejaculation protocol that reliably produced semen samples from individuals of this taxon. The importance of the rectal probe in electroejaculation has long been recognized, and probe modifications have been required in the past to better fit the unusual anatomy of some nondomestic species.[6] In fact, this is not the first report of a custom-built probe for a rhinoceros. The use of an inflatable model on an unanesthetized animal was reported in 1990, but the outcome was unsuccessful.[21] More recently, the use of a handheld probe has proven quite effective in the white rhinoceros.[5] The probe used in this study was relatively easily and inexpensively assembled using products available at most home building stores, and the design may serve as a template for those working with other species for which standard linear probes are ineffective for similar reasons. Although the probe models were tested in a greater one-horned rhinoceros until the design was deemed satisfactory, the final version of the probe also appropriately fit the black and white rhinoceros species.

In addition to the probe, the technique used during the procedure was vitally important to the eventual outcome. Becoming familiar with the animals' responses, what to expect, and how to change things if the desired response was not achieved were all critical during the procedure. The primary technical challenge was trying to maintain the probe in precisely the right position within the rectum during electrical stimulation when the animal's muscles contracted. At the higher voltages, doing so required strength and significant effort by the technician.

Both penile and rectal massage were also important components of the procedure. Penile massage both during and between series of stimuli appeared to induce expulsion of fluid fractions. Rectal massage between series of stimuli may have been helpful in promoting emissions and the pooling of semen in the pelvic urethra. Although this phenomenon was not confirmed by ultrasonography during the procedure as it has been in other studies,[20] the collection of larger-volume, more concentrated sample fractions shortly after the resumption of

stimulation suggested that sample pooling had taken place during the massage period.

Penile erection was often, but not always, achieved during the procedure and was not essential to the collection of a good-quality fraction. Stimulation at the lower voltages early in the procedure often resulted in penile retraction. Conversely, later in the procedure at higher voltages, the penis typically became more erect, with retraction more likely during rest intervals.

The total number of spermatozoa contained in ejaculates collected in this study far exceeded those previously reported. Historically, sperm numbers were low, in part, because ejaculate volume did not exceed 45 ml.[15,20] More recently, electroejaculation using a handheld probe produced samples averaging 80 ml in volume in the white rhinoceros, but mean total sperm numbers ranged from 1.1 to 2.8 $\times$ 10^9 for poor-quality and high-quality samples, respectively.[5] Although mean ejaculate volume was only slightly higher in this study, mean total sperm numbers were at least 10-fold greater, ranging from 10.5 to 37.1 $\times$ 10^9 for ejaculates containing poor- and good-quality fractions, respectively. Furthermore, the electrical stimulation required to obtain the larger-volume samples reported in previous studies was as high as 20–29 V[5,15] with conductance reaching up to 900 mA.[5] In this study, the highest electrical stimulation required was 10 V, and the conductance typically reached 400 mA. These differences indicate that the custom probe used in this study was effective in making good, targeted contact with the male rhinoceros's rectal lining and stimulating in the region necessary to elicit the desired response.

Regardless of the large fluid volume collected during the procedure, only fractions of it contained spermatozoa. Typically, the initial fluid emissions were aspermic or very dilute, especially if fluid was collected early in the procedure. As the stimulation increased, sample fractions usually became more concentrated with spermatozoa until reaching a maximal concentration and then becoming more dilute. However, this pattern could vary substantially. In some cases, several series of stimuli were delivered before any fluid was produced. On these occasions, the early fractions could be highly concentrated with spermatozoa.

Information regarding sperm quality following semen collection attempts in the rhinoceros has been nonexistent[20] to minimal[15,21] in the past. However, a recent study on the white rhinoceros provides useful information on ejaculate sperm motility and morphology following electroejaculation.[5] Data herein provide the first detailed information regarding the characteristics of rhinoceros electroejaculates. Interestingly, sample volume, osmolality, sperm motility, and morphology are very similar to those reported for semen samples collected from a female Sumatran rhinoceros after natural mating.[13] Although postcoital samples appeared slightly more acidic (pH = 7.9) and dilute (24.5 $\times$ 10^6/ml), both characteristics were likely influenced by the environment from which the samples were collected.

Unlike its equid relatives, the rhinoceros did not appear to ejaculate distinctly different gel and sperm-rich fractions, although there was some minor variation in the viscosity among fractions collected. It is possible that this result was technique-related, because electroejaculation can affect the viscosity of samples produced. Regardless, rhinoceros ejaculate characteristics compared favorably to those of horses, ponies, and zebras. In general, rhinoceros electroejaculates contained more total spermatozoa than ejaculates of other equids collected by manual stimulation, AV, or chemical induction.[3,4,9–11] The total volume collected was also greater for the rhinoceros than for the horse or pony,[3,9–11] but less than that reported for a mature Grevy's zebra following manual stimulation.[4] Interestingly, although there were no distinct gel and gel-free fractions collected from the rhinoceros, the mean volume of good-quality fractions was similar to that of the gel-free fractions collected from horses ejaculating naturally into an AV or following manual stimulation or chemical induction.[9–11] One consistent difference between rhinoceros ejaculates and those of the other three equids was the pH, which appeared to be more basic (8.5) for the rhinoceros than for the horse (6.7–7.5),[9,10] pony (6.8–6.9),[11] and zebra (7.7).[4]

The variability in sample quality from one ejaculate fraction to the next was considerable, and it was absolutely essential that the collection cup be changed frequently to prevent contamination of a high-quality fraction with a fraction of low quality. The data did not reveal one factor or group of factors that could be used to differentiate ejaculate fractions containing good-quality, concentrated, motile spermatozoa versus those with dilute, immotile spermatozoa. Therefore, speculations that pH or osmolality differences would be associated with fraction quality were not supported.

One challenge to electroejaculation in many species is contamination of the ejaculate with urine. The same appears true for the rhinoceros. However, whereas some fractions of the ejaculate might contain urine, those fractions collected before and after the urine-contaminated sample could contain high-quality spermatozoa. There was no specific pattern

noted with urine-contaminated fractions that might indicate a cause-and-effect relationship with any particular aspect of the procedure (i.e., a certain placement of the probe or voltage used). Although spermatozoa in urine-contaminated samples could be motile, quality was definitely diminished.

A more interesting and unexpected occurrence was the presence of red blood cells in several ejaculate fractions. Hemospermia was noted in multiple males but did not occur during every electroejaculation attempt in those males. Furthermore, good-quality ejaculate fractions devoid of RBC could be collected before or after the fractions containing RBC. Hemospermia has been reported in stallions ejaculating naturally into an AV, but it is not a common condition and is usually caused by irritation of the urethral process because of trauma, infection, or neoplasia.[1,19] Although RBC can be spermicidal, low-grade hemospermia may be managed by adding an extender to dilute the RBC.[2] Hemospermia in the rhinoceros could be considered primarily low-grade because samples often just contained a faint pink hue. Therefore, it is not surprising that the spermatozoa often were viable and motile in the sample fractions containing some RBC. The cause of hemospermia in the rhinoceros is unknown and its intermittent expression was puzzling.

Sperm structural abnormalities were common across all study animals and species, with bent midpieces and bent or coiled tails the most prevalent morphological anomalies. These findings are similar to those previously reported for the white rhinoceros in which the percentage of morphologically intact spermatozoa following electroejaculation also was relatively low (52.8–75.8%).[5] However, their most prevalent abnormality was a detached tail.

Initially, the high percentage of abnormal spermatozoa was considered a potential product of the electroejaculation procedure, the theory being that the unrefined technique was causing disproportionate ejaculation of glandular fluids resulting in pH or osmolality shifts that shocked spermatozoa during the ejaculatory process. Both pH and osmolality can have profound effects on sperm quality,[8] but the similarity of both characteristics in ejaculate fractions containing good-quality and poor-quality sperm samples argues against either one being the culprit. Furthermore, spermatozoa collected from the epididymides of the black rhinoceros that was euthanized immediately following electroejaculation were inferior in quality to those collected during the electroejaculation procedure, and only 71% of the spermatozoa were morphologically normal without any exposure to glandular secretions. Finally, even following natural ejaculation during mating, a high number of structurally abnormal spermatozoa have been collected in a Sumatran rhinoceros.[13] Therefore, the presence of structurally abnormal spermatozoa may not be unusual in rhinoceros ejaculates.

Because most samples collected in this study were from greater one-horned rhinoceroses, it is possible that this species, or individuals thereof, are teratospermic. The greater one-horned rhinoceros once was reduced to very low numbers and could have experienced genetic bottlenecks. For example, the population in Kaziranga National Park was estimated at just 10–12 individuals before bolstered protection and management of the park enabled the population to rebound.[12] Therefore, a loss of genetic diversity could be associated with a species-wide reduction in sperm quality, as has been reported in other species.[14,16,17] However, a larger sample size will need to be examined before any conclusions can be made.

The criteria for classifying good-quality samples ($\geq$60% motile and $>20 \times 10^6$ spermatozoa/ml) were based on several pieces of information. First, these values were in line with those reported for a natural ejaculate collected from a female rhinoceros postmating.[13] Second, the percentage of motile spermatozoa needed to be high enough that an expected reduction of 10% following cryopreservation[13] would still produce a sample exhibiting 50% motility post-thaw. Finally, the sample had to be concentrated enough initially that the addition of the cryodiluent would not render it too dilute to evaluate accurately post-thaw. Despite the challenges of the procedure and some of the sperm quality issues encountered during this study, several samples met the criteria and were suitable for cryopreservation and future use in AI attempts. Similar to findings with the white rhinoceros,[5] there were no apparent seasonal effects on ejaculate quality: good-quality fractions were collected in August, November, December, February, April, May, and June.

In summary, a uniquely designed rectal probe and refined procedural techniques have made electroejaculation a relatively reliable and safe method of semen collection in the rhinoceros. However, the quality of sample fractions collected is highly variable, and the procedure does not always result in a good-quality fraction despite concerted efforts to change collection cups frequently during the procedure. Therefore, a positive result from electroejaculation is helpful in assessing male rhinoceros fertility, but a negative result is meaningless and should not, by itself, be used to diagnose infertility. Although there still is room for improvement, elec-

troejaculation in the rhinoceros has progressed beyond the experimental stage and now is producing quality semen samples suitable for sperm banking and assisted reproduction procedures. Therefore, it is now appropriate to integrate this technology into the intensive management plan for the long-term preservation of this taxon.

Acknowledgments: We are extremely grateful to all the extra hands that helped with these procedures, including keeper, veterinary, scientific, and animal management staff at all three facilities. In particular, the staff at The Wilds should be commended for their commitment to this project from the very beginning when probe designs were being tested and redesigned. Their patience, encouragement, and participation in this study were essential to its eventual success. Additionally, we sincerely thank Mark H. Lawry and Tom Rocklin for their computer modeling work that created the 3D images of the probe design. This study was funded, in part, by grants from the International Rhino Foundation and Morris Animal Foundation (#DO3ZO-58).

LITERATURE CITED

1. Bedford, S. J., S. M. McDonnel, E. Tulleners, D. King, and P. Habecker. 2000. Squamous cell carcinoma of the urethral process in a horse with hemospermia and self-mutilation behavior. J. Am. Vet. Med. Assoc. 216: 551–553.

2. Blanchard, T. L. 1987. Use of a semen extender containing antibiotics to improve the fertility of a stallion with seminal vesiculitis due to *Pseudomonas aeruginosa*. Theriogenology 28: 541–546.

3. Crump, J., and J. Crump. 1989. Stallion ejaculation induced by manual stimulation of the penis. Theriogenology 31: 341–346.

4. Crump, J. P., and J. W. Crump. 1994. Manual semen collection from a Grevy's zebra stallion (*Equus grevyi*), onset of sperm production, semen characteristics, and cryopreservation of semen with a comparison to the sperm production from a Grant's zebra stallion (*Equus burchelli boehmi*). Theriogenology 41: 1011–1021.

5. Hermes, R., T. B. Hildebrandt, S. Blottner, C. Walzer, S. Silinski, M. L. Patton, G. Wibbelt, F. Schwarzenberger, and F. Goritz. 2005. Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum*, *C.s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation. Theriogenology 63: 219–238.

6. Herrick, J. R., M. K. Campbell, and W. F. Swanson. 2002. Electroejaculation and semen analysis in the La Plata three-banded armadillo (*Tolypeutes matacus*). Zoo Biol. 21: 481–487.

7. Hildebrandt, T., R. Hermes, F. Schwarzenberger, C. Walzer, S. Silinski, A. Schnorrenberg, and F. Goritz. 2002. A newly developed artificial insemination technique in African rhinoceroses. Proc. Am. Assoc. Zoo Vet. 213–215.

8. Makler, A., R. David, Z. Blumenfield, and O. S. Better. 1981. Factors affecting sperm motility VII. Sperm viability as affected by change of pH and osmolarity of semen and urine specimens. Fertil. Steril. 36: 507–511.

9. McDonnell, S. M., and C. C. Love. 1990. Manual stimulation collection of semen from stallions: training time, sexual behavior and semen. Theriogenology 33: 1201–1210.

10. McDonnell, S. M., and M. J. Odian. Imipramine and xylazine-induced ex copula ejaculation in stallions. Theriogenology 41: 1005–1010.

11. McDonnell, S. M., and R. M. O. Turner. 1994. Post-thaw motility and longevity of motility of imipramine-induced ejaculates of pony stallions. Theriogenology 42: 475–481.

12. Oberai, C. P., and B. S. Bonal. 2002. Kaziranga the Rhino Land. B. R. Publishing Corporation, Delhi, India. P. 24.

13. O'Brien, J. K., and T. L. Roth. 2000. Post-coital sperm recovery and cryopreservation in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) and application to gamete rescue in the African black rhinoceros (*Diceros bicornis*). J. Reprod. Fertil. 118: 263–271.

14. O'Brien, S. J., M. E. Roelke, L. Marker, A. Newman, C. A. Winkler, D. Meltzer, L. Colly, J. F. Evermann, M. Bush, and D. E. Wildt. 1985. Genetic basis for species vulnerability in the cheetah. Science 227: 1428–1434.

15. Platz, C., S. W. J. Seager, and M. Bush. 1979. Collection and analysis of semen from a black rhinoceros. J. Am. Vet. Med. Assoc. 175: 1002–1004.

16. Roelke, M. E., J. S. Martenson, and S. J. O'Brien. 1993. The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Curr. Biol. 3: 340–350.

17. Roldan, E. R., J. Cassinello, T. Abaigar, and M. Gomendio. 1998. Inbreeding, fluctuating asymmetry, and ejaculate quality in an endangered ungulate. Proc. R. Soc. Lond. B Biol. Sci. 265: 243–248.

18. Roth, T. L., and J. L. Brown. 1999. Is there any rhyme or reason to rhino reproduction? A summary of reproductive characteristics, species specificities and challenges for the future. Proc. Am. Assoc. Zoo Vet. 97–99.

19. Schumacher, J., D. D. Varner, D. Schmitz, and T. L. Blanchard. 1995. Urethral defects in geldings with hematuria and stallions with hemospermia. Vet. Surg. 24: 250–254.

20. Shaffer, N., W. Bryant, D. Agnew, T. Meehan, and B. Beehler. 1998. Ultrasonographic monitoring of artificially stimulated ejaculation in three rhinoceros species (*Ceratotherium simum*, *Diceros bicornis*, *Rhinocero unicornus*). J. Zoo Wildl. Med. 29(4): 386–393.

21. Shaffer, N. E., B. Beehler, R. S. Jeyendran, and B. Balke. 1990. Methods of semen collection in an ambulatory greater one-horned rhinoceros (*Rhinoceros unicornis*). Zoo Biol. 9: 211–221.

22. Shaffer, N. E., Z. Zainal-Zahari, M. S. M. Suri, M. R. Jainudeen, and R. S. Jeyendran. 1994. Ultrasonography of the reproductive anatomy in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). J. Zoo Wildl. Med. 25: 337–348.

23. Stoops, M. A., R. D. Pairan, and T. L. Roth. 2004. Follicular, endocrine and behavioral dynamics during the estrous cycle of the Indian rhinoceros. Reproduction 128: 843–856.

Received for publication 1 March 2005