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Factors impacting the success of post-mortem sperm rescue in the rhinoceros

T.L. Roth[a,*], M.A. Stoops[a], T.R. Robeck[b], J.K. O'Brien[b]

[a] Center for Conservation and Research of Endangered Wildlife (CREW), Cincinnati Zoo & Botanical Garden, Cincinnati, OH 45220, USA
[b] SeaWorld and Busch Gardens Reproductive Research Center, SeaWorld Parks and Entertainment, 2595 Ingraham St., San Diego, CA 92109, USA

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ABSTRACT

The goal of this study was to identify factors that influenced the ability to successfully rescue sperm post-mortem from rhinoceroses maintained in North American zoos. Factors considered included procedural technicalities, individual rhinoceros characteristics and timing. Gross testicular pathology was noted in 17.4% of males (4/23) but did not impact sperm recovery except in one case of azoospermia (4.3%). Of the males in which sperm recovery was attempted ($n = 21$), 62% yielded quality samples considered adequate for cryopreservation (≥30% motility with ≥2.0 forward progressive status). A high percentage of males (70.6%; 12/17) from which reproductive tissue was removed and cooled ≤4 h after death yielded quality sperm samples, whereas only 25% (1/4) of males from which tissue was removed >4 h after death yielded quality samples. Quality samples were recovered 1–51 h post-mortem from rhinoceroses 8 to 36 years old. Neither type of illness (prolonged or acute), or method of death (euthanasia or natural) affected the ability to harvest quality samples ($P > 0.05$). The Indian rhinoceros yielded significantly more sperm on average (40×10^9) than the African black rhinoceros (3.6×10^9; $P < 0.01$) and the African white rhinoceros (3.2×10^9; $P < 0.05$). Across all species and samples assessed ($n = 11$), mean post-thaw sperm motility (41%), was only 15% less than pre-freeze motility (56%) and only decreased to 22% during the 6 h post-thaw assessment period. Rhinoceros sperm rescue post-mortem is relatively successful across a wide range of variables, especially when tissues are removed and cooled promptly after death, and should be considered standard practice among zoos.

1. Introduction

Given the increasing anthropogenic pressures on wildlife around the globe coupled with the challenges of climate change, there is a growing realization that many species may be driven to extinction by the end of the 21st century (Barnosky et al., 2011). Faced with this reality, conservation scientists are acknowledging the need to focus more attention on cryopreserving genetic resources from extant species while the opportunity still exists (Wildt, 2000; Harnal et al., 2002; Fickel et al., 2007). Relatively small-scale, yet still significant efforts to bank wildlife materials including sperm, embryos and cell lines have been ongoing in several zoo-associated labs for over two decades. Therefore, it seems an opportune time to evaluate these somewhat limited, opportunistic efforts in an attempt to capitalize on what we can learn from existing results and improve success and efficiency in the future.

* Corresponding author.
E-mail address: terri.roth@cincinnatizoo.org (T.L. Roth).

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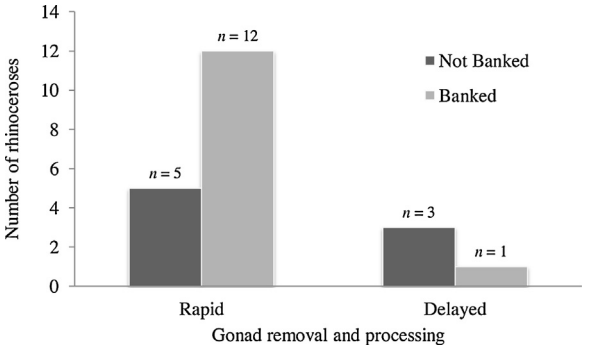

Fig. 1. Number of successful (banked) versus failed (not banked) rhinoceros sperm rescue attempts following rapid (<4 h post-mortem) tissue removal and cooling or delayed (>4 h post-mortem) tissue removal and cooling.

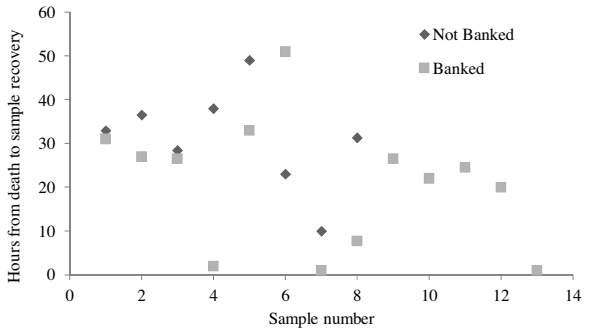

Fig. 2. Scatter plot demonstrating no strong relationship between the interval from death to sperm recovery (up to 51 h post-mortem) and the ability to harvest quality samples ($n = 13$ successful/banked and $n = 8$ unsuccessful/not banked).

Arguably, the rhinoceros is one taxon that stands out as a primary candidate for concerted genetic banking efforts. First, all rhinoceros species are currently or have historically come very close to extinction (Milliken et al., 2009). Furthermore, even those species that have made an impressive recovery are now severely threatened by the recent and rapid escalation in poaching activities (Milliken, 2014). In addition, rhinoceroses give birth to singletons after a lengthy gestation (16 months), rendering populations incapable of rebounding quickly when numbers drop to perilously low levels. Although zoos accredited by the Association of Zoos and Aquariums (AZA) currently maintain three rhinoceros species within their facilities (the African white rhino, *Ceratotherium simum*; the African black rhino, *Diceros bicornis*; and the Indian rhino, *Rhinoceros unicornis*), and all three breed successfully in captivity, within each exists a substantial number of individuals that have never reproduced (Guldenschuh and von Houwald, 2009; Christman, 2011a,b). Unless their genetic potential is preserved for future use, each individual's unique genetic milieu will be lost at death.

Although sperm and embryo banking is often touted as a means of ensuring the future for endangered species, the reality is that the material banked today must be functionally competent when thawed in the future, and there must be proven protocols available for producing pregnancies in the sperm and embryo recipients, or the limited, valuable banked resources are likely to be exhausted to no avail. In the case of the rhino, proof already exists that cryopreserved semen is fully functional post-thaw after many years in liquid nitrogen, and protocols have been established for producing offspring following artificial insemination with frozen–thawed sperm in both Indian rhinoceroses and white rhinoceroses (Stoops et al., 2007; Hermes et al., 2009; respectively). Therefore, sperm banking in the rhinoceros is based on an existing solid scientific foundation and is likely to yield fully functional samples capable of producing live young in the future. However, inherent in efforts to recover quality sperm samples for cryopreservation post-mortem are logistical and biological challenges that need to be identified so that methodologies for overcoming these obstacles can be employed to increase the odds of success.

Several challenges inherent to post-mortem sperm rescue have been investigated in other species and provide a precedent for this study. In Iberian red deer, both season and time of rescue post-mortem impacted sperm recovery efforts (Martinez-Pastor et al., 2005). Similarly, time of rescue relative to time of death significantly impacted sperm quality in the Spanish ibex (Fernández-Santos et al., 2011). In many species including the horse, ram, boar, deer, dog and mouse, tissue storage and/or transport at a cooler environmental temperature facilitated the harvest of quality sperm many hours post-mortem (Kikuchi et al., 1998; An et al., 1999; Yu and Leibo, 2002; Soler et al., 2005; Lone et al., 2011; Monteiro et al., 2013). Despite the difficulties, sperm collected and cryopreserved post-mortem and later used for artificial insemination has already produced offspring in several nondomestic species including the chimpanzee (Kusunoki et al., 2001), marmoset (Morrell et al., 1998), eland (Bartels et al., 2001) and Spanish ibex (Santiago-Moreno et al., 2006), proving the fertility of such samples.

The overall goal of this study was to retrospectively analyze data collected over a 16 year period to identify factors associated with the ability to rescue sperm from rhinoceroses post-mortem. Our three hypotheses were that the following factors impact our ability to rescue quality sperm samples post-mortem from the rhinoceros: (1) technical and temporal differences in post-mortem reproductive tissue removal, processing, packaging and shipping; (2) variation in rhinoceros characteristics (age, type and length of illness, method of death); and (3) environmental factors (temperature, season).

2. Materials and methods

2.1. Animals

A total of 23 mature male rhinoceroses representing 4 species and maintained at 13 different AZA-accredited zoos were opportunistically included in this study over a 16 year period (1998- 2014). The majority of the males were African black rhinoceros ($n = 14$), followed by Indian rhinoceros ($n = 5$), African white rhinoceros ($n = 3$) and a single Sumatran rhinoceros. The range in ages, mean and median age for each species were (8–32 year; 20.9 year; 21

year), (15–43 year; 25.6 year; 32 year), (10–35 year; 25.7 year; 20 year) and (33 year; 33 year), respectively.

2.2. Reproductive tissue removal, processing and shipping

The protocol for reproductive tissue removal, processing and shipping was distributed to all AZA accredited zoos with rhinoceroses in their collections. In many cases, the zoo staff was able to follow the protocol exactly as written, but in other cases, there were some deviations from the protocol. For those that followed the protocol, the reproductive tisse (testes, epididymides and vas deferens) was removed from the rhinoceros as soon as possible after the rhino's death, preferably within 1–2 h. The attached vas deferens were ligated with plain suture material to avoid sperm leakage during transport. Reproductive tissue was placed in zip-lock plastic bags with a few pieces of gauze soaked in saline or phosphate buffered saline to keep tissue moist. Baggies were then wrapped in a towel or in multiple layers of paper towels to prevent direct contact of tissue with the ice packs in the Styrofoam shipping container. One to two ice packs were included in the shipping container to maintain a temperature of approximately 5 °C during shipment. The package was shipped overnight for next day morning delivery.

2.3. Sperm harvesting and assessment

Upon arrival at the reproductive laboratory, the package was opened and any observations about the packaging not in accordance with the recommended protocol were noted, as was the temperature and apparent health and integrity of the tissue. Testes were examined grossly and measured for length and width using calipers. Any morphological abnormalities were noted (size, firmness, presence of a mass). Vas deferens and cauda epididymides were then dissected from surrounding connective tissue and blood vessels. When an intact portion of the vas deferens was included with the reproductive tissues, sperm were flushed from it into a Petri dish by inserting a small needle (23–25 gauge) into one end and injecting warmed 37 °C Tyrode's albumen, lactate, pyruvate (TALP) medium or a standard equine semen extender (EQ; Martin et al., 1979; O'Brien and Roth, 2000). Most sperm were collected by slicing the tubules of the cauda epididymis numerous times with a scalpel blade and extruding the thick sperm suspension by squeezing the tissue with forceps. The scalpel blade or a pipettor was then used to collect the sperm-rich fluid where it had oozed out of the tubules and transfer it into a Petri dish containing a small volume (~0.5–1 mL) of TALP or EQ. Although TALP was initially used during sperm collection, in later years, EQ was the only medium used for sperm collection and processing for cryopreservation. A small aliquot of the sperm suspension was examined microscopically for initial percent motility (proportion of sperm exhibiting motility) and progressive motility rating (scale of 0–5; 0 = no forward progression; 5 = all sperm exhibiting rapid forward progression). If motility was poor (<30%), sperm viability was assessed using eosin-nigrosin staining and if >50% of the sperm contained intact plasma membranes, the sample was held at 37 °C or at 4 °C for 1–2 h and assessed intermittently to determine if motility had improved. If at least 30% of the sperm were motile and the progressive motility rating was ≥2.0, the sample was processed for cryopreservation.

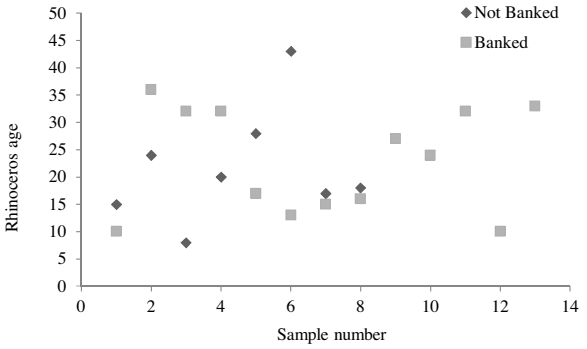

Fig. 3. Scatter plot demonstrating quality samples can be recovered from rhinoceroses 10–36 year of age.

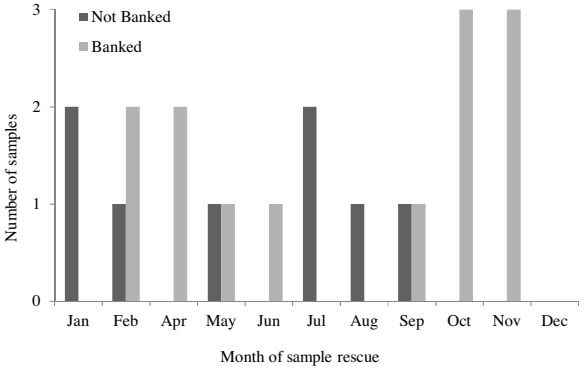

Fig. 4. Number of successful (banked, $n = 13$) versus failed (not banked, $n = 8$) sperm rescue attempts by month.

2.4. Sperm cryopreservation

Sperm samples that met the criteria for cryopreservation (≥30% motility and ≥2.0 progressive motility rating) were cryopreserved according to methods described in detail by O'Brien and Roth, 2000; and Stoops et al., 2010; with very few minor modifications. Briefly, samples initially collected in TALP were diluted 1:1 with EQ. Samples were transferred to 15 ml centrifuge tubes, final volume was recorded, and sperm concentration was determined using a hemocytometer. Samples were typically further diluted with EQ extender as necessary to achieve a final sperm concentration of ~100–200 × 10^6 sperm/ml in the straws. Final sample volume was measured and a similar volume of EQ extender containing 10% glycerol was placed in a second 15 ml centrifuge tube. All dilutions and assessments were conducted at room temperature after the initial harvest with pre-warmed medium (37 °C). Tubes of semen and EQ with 10% glycerol were placed in a room temperature water bath that was transferred into a cold room (4 °C) to cool for ~1.5 h during which the

temperature of the bath was checked frequently and ice was added to the water bath to facilitate cooling to ~4–6 °C. Samples were then diluted with an equal volume of the EQ containing 10% glycerol in a step wise fashion (25%, 25% and 50% of volume) at 20 min intervals for a final 5% glycerol concentration in the sperm sample. Samples were then allowed to equilibrate for an additional 30–60 min prior to freezing. During the equilibration period, ½ cc straws were filled with sample in the cold room and sealed with critoseal (Thermo Fisher Scientific Inc., Waltham, MA USA 02451) or ball bearings (Minitube of America, Verona, WI, USA 53593). Straws were placed in goblets on canes (6–8 straws/goblet) and the canes placed in the canister of a dry shipper. After all straws were filled, the canister containing the canes was lowered in one swift motion into the dry but fully charged dry shipper and the top was put in place to cover the neck opening. After 10 min, liquid nitrogen was poured into the dry shipper, thereby effectively plunging the semen straws. Post-thaw sperm motility was assessed for many of the banked samples by thawing a test straw 1–2 weeks after cryopreservation. To thaw the semen, the straw was wafted in air for 10 s before being plunged into a 37–38 °C water bath and shaken for another 20 s. The straw content was then emptied into a microcentrifuge tube and an aliquot evaluated subjectively immediately and 6 h after thawing for percent sperm motility and progressive motility rating. Most samples were also assessed for viability and acrosome integrity immediately post-thaw.

2.5. Statistical analysis

A Fischer's exact 2-tailed test was used to compare sperm quality results from samples: (1) harvested from tissues cooled rapidly (<4 h) post-mortem versus >4 h post-mortem, (2) harvested from euthanized males versus males that died of natural causes, and (3) harvested from males that died acutely or following a prolonged illness. In addition, a one-way analysis of variance followed by a post-hoc Scheffé test were used to compare the total number of sperm recovered from African black, African white and Indian rhinos and to compare percent sperm motility at 6 h post-thaw between samples collected in TALP versus EQ and between those collected from rhinos who died naturally or were euthanized. Finally, a Pearson correlation coefficient test was employed to determine if rhinoceros age or interval from death to tissue processing were associated with percent sperm motility in the recovered sample prior to cryopreservation or at 6 h post-thaw.

3. Results

3.1. Gross pathology

Of the 23 males from which testes were received, gross evidence of pathology was noted in four (17.4%) in the form of a mass in one testis or visible atrophy of one or both testes. In three of these four males, sperm were still being produced, and samples that met the cryopreservation criteria were recovered from two of these males. Samples from both cauda epididymides of the third male (black rhino) were aspermic (1/23 total; 4.3%) and the harvested

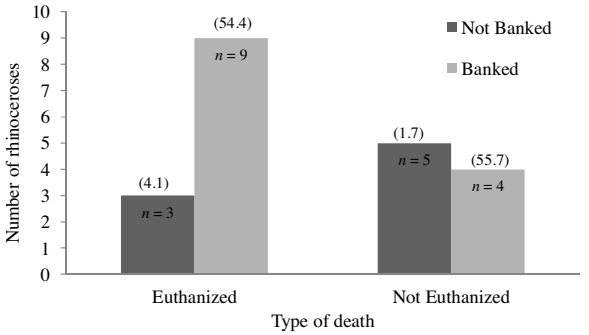

Fig. 5. Number of successful (banked) versus failed (not banked) rhinoceros sperm rescue attempts following death by euthanasia versus natural causes. Number above each column represents mean total percent sperm motility for each sample group.

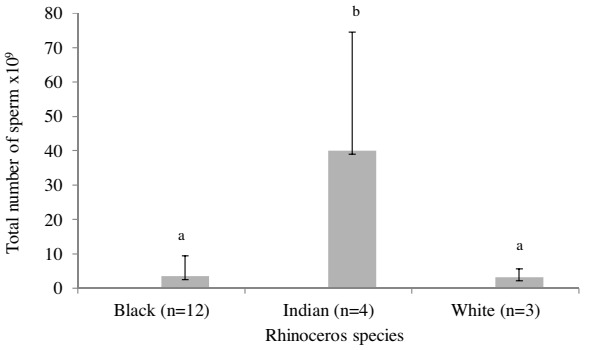

Fig. 6. Mean (+SD) total number of epididymal sperm harvested from black rhinos, Indian rhinoceroses and white rhinoceroses post-mortem. Columns with different superscripts differ ($P < 0.05$).

sperm from the fourth (Indian rhino) did not meet the cryopreservation criteria. Two rhinoceroses were removed from the data base for subsequent analyses: the aforementioned aspermic black rhinoceros and the spermic Indian rhinoceros for which sperm rescue attempts were aborted after the initial testicular examination and sperm assessment.

3.2. Gonad removal, processing and packaging

Precise data on the time from death to gonad removal were rarely available, however, general time frames could be determined in most cases. Reproductive tissue removal and/or cooling was considered rapid if it occurred within approximately 4 h of death and delayed if it occurred >4 h after death. Rhinoceroses subjected to pre-planned euthanasia were all presumed to fall into the rapid category and any animals that died overnight were presumed to be in the delayed category when more specific information was not provided. Rapid removal/cooling was conducted for the majority of the 21 rhinoceroses ($n = 17$) with delayed removal/cooling comprising just 19% ($n = 4$; Fig. 1). Although 70.6% of the samples from tissues that were cooled rapidly were banked, and only 25% of the samples from tissues not cooled rapidly were banked, given the small, imbalanced sample sizes, the statistical analysis was

not powerful and did not indicate a significant difference in sperm quality between the two groups ($P = 0.25$).

For three of the five cases in which reproductive tissue was removed and cooled rapidly post-mortem but quality sperm samples were not harvested, a deviation from the processing/packaging/shipping protocol could be identified as the probable cause (i.e., 10 ice packs in shipping container, package in transit for 2 days, reproductive tissue stored in saline at 4 °C overnight prior to shipping). However, in two cases, tissue removal, processing and shipping were carried out exactly as specified in the protocol but samples were non-viable upon harvest (0% and 11% plasma membrane intact sperm).

Time from rhinoceros death to sperm cell harvest varied from 1–51 h with the majority ($n = 14$) occurring between 20–38 h post-mortem (Fig. 2). Samples meeting the sperm banking criteria could be collected from tissues up to 51 h post-mortem as long as tissues were processed according to the protocol. There was no significant correlation between interval from death to processing and percent sperm motility in the recovered sample ($P = 0.33$).

3.3. Rhinoceros characteristics

Of the 21 attempts to rescue and cryopreserve sperm, 13 (62%) were successful in yielding samples that met the sperm banking criteria (Fig. 2). All four rhinoceros species were represented in these 13 samples (black rhino, $n = 7$; white rhino, $n = 3$; Indian rhino, $n = 2$; and Sumatran rhinoceros $n = 1$).

3.3.1. Age

Spermic samples were harvested from tissues of the youngest and the oldest rhinoceroses in the study (8 and 43 year old, respectively; Fig. 3). A high percentage of the samples (70%, 7/10) recovered from tissues of rhinoceroses 20–36 year of age met the quality criteria for sperm banking. There was no correlation between rhinoceros age and percent sperm motility in samples recovered post-mortem ($P = 0.89$).

3.3.2. Method of death

Tissues were recovered from a total of 12 rhinoceroses that were euthanized for declining medical conditions by iv injection of Euthasol® (pentobarbital sodium and phenytoin sodium; Virbac AH, Inc., P.O. Box 162059, Fort Worth, TX 76161) and from 9 rhinoceroses that died of natural causes. Of the rhinoceroses euthanized, 75% ($n = 9$) yielded quality sperm samples that met the banking criteria and 25% ($n = 3$) did not (Fig. 5). Of the rhinoceroses that died naturally, only 44.4% ($n = 4$) yielded sperm samples that met the banking criteria and 55.6% ($n = 5$) did not (Fig. 5). These differences were not statistically significant ($P = 0.20$).

3.3.3. Cause of death

Due to a number of factors including the lack of detailed information received regarding each rhino's health status leading up to mortality, the difficulty in determining ultimate cause of death in some cases and the variety of illnesses reported, it was not possible to find any associations between cause of death and ability to rescue quality sperm. If rhinoceroses euthanized due to severe arthritis, or those that died from impaction or colic were not considered to have died from an extended illness, and rhinoceroses that died from renal failure, hemochromatosis, or following symptoms of inappetence, weight loss, lethargy for more than 10 days were considered to have died from a lengthy illness, there were approximately equal numbers of rhinoceroses that died acutely ($n = 9$) and died after an extended illness ($n = 8$) and several whose cause of death was unknown ($n = 6$). There did not appear to be an association with sample quality and acute versus extended illness. Of the rhinoceroses that yielded quality sperm samples ($n = 13$), five died acutely, five from lengthy illnesses and three were unknown. Similarly, of the rhinoceroses that did not yield quality sperm samples ($n = 8$), three died acutely, three from lengthy illness and two were unknown.

3.4. Environmental factors

Sperm rescue attempts occurred throughout the year with at least one harvest conducted each month except during the months of December and March (Fig. 4). Samples meeting the banking criteria could be harvested throughout the year suggesting there was no seasonal impact. However, none of the five harvest attempts in January, July and August yielded quality sperm samples.

3.5. Additional findings

3.5.1. Total sperm production by species

Unexpected data emerging from this study that is of interest in light of previous publications about semen collection in rhinos, was the difference in total sperm yield from different rhinoceros species. Although the data base is limited given the small sample size for Indian rhinoceroses and African white rhinos, it was hard to ignore the apparent 10-fold increase in mean numbers of sperm harvested from Indian rhinoceroses compared with the totals harvested from any other rhinoceros species (Fig. 6). Species' differences in total sperm production were statistically significant ($P = 0.003$) with the Indian rhinoceros producing significantly more sperm than the African black rhinoceros ($P < 0.01$) and the African white rhinoceros ($P < 0.05$), but no difference between the two African rhinoceros species ($P > 0.05$). Individual variation within each species was significant with sperm harvest numbers ranging from 0.3–21 $\times 10^9$ in African black rhinos, 8.2–85 $\times 10^9$ in Indian rhinoceroses and 0.6–5.6 $\times 10^9$ in African white rhinos. The single Sumatran rhinoceros was excluded from the species comparison analysis but sperm recovery from this male was quite low (130 $\times 10^6$ total sperm).

3.5.2. Quality of sperm sample

Of the 13 samples that were cryopreserved in this study, 11 have been assessed for post-thaw sperm motility, and of those, 8 also were evaluated for viability and acrosome integrity. Although pre-freeze percent sperm motility varied substantially across rhinoceroses ranging from 30% to 85%, a decrease of only 15% on average was observed when comparing pre-freeze to initial post-thaw mean motility values (Fig. 7). The reduction in total sperm motility

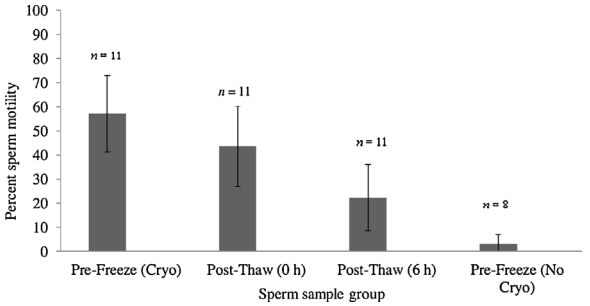

Fig. 7. Mean (±SD) percent motile sperm in pre-freeze and post-thaw rhinoceros sperm rescue samples that were banked, and that for samples deemed too poor quality to bank.

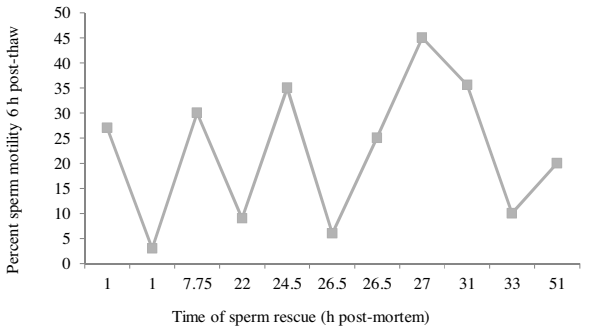

Fig. 8. Percent sperm motility 6 h post-thaw for rhinoceros sperm samples ($n = 11$) rescued 1–51 h post-mortem. Time of rescue was not correlated with percent sperm motility 6 h post-thaw ($R = .097$).

pre-freeze versus immediately post-thaw was fairly similar among rhinoceros species: (black rhino, $n = 6$, 53% versus 38%; white rhino, $n = 2$, 80% versus 63%; Indian rhino, $n = 2$, 50% versus 45%; and Sumatran rhino, $n = 1$, 50% versus 38%). Percent sperm motility in samples that were not cryopreserved was much lower (range, 0–20%). At 6 h post-thaw, mean sperm motility across species was still at 22% (Fig. 7) and did not differ for samples initially recovered in TALP versus EQ ($P = 0.18$) or between samples collected from rhinos that died of natural causes or were euthanized ($P = 0.36$). Furthermore, there was no correlation ($R = .097$) between time of sample recovery and the percentage of motile sperm 6 h post-thaw (Fig. 8). Percentage of viable and acrosome intact sperm varied significantly among samples ranging from 32–92% viable and 29–97% intact immediately post-thaw.

4. Discussion

Several case reports of post-mortem sperm rescue in the rhinoceros have been reported (Williams et al., 1995; Lubbe et al., 1999; O'Brien and Roth, 2000). However, this study is the first robust retrospective analysis of factors impacting post-mortem sperm rescue and cryopreservation success in the rhinoceros. It was encouraging to learn that across all conditions, viable samples meeting the cryopreservation criteria were successfully harvested from over half the total attempts, indicating that sperm rescue from rhinoceroses maintained in North American zoos is certainly a worthwhile endeavor.

Because a substantial number of captive rhinoceroses do not contribute to their species' population via successful reproduction, the fertility of both male and female rhinoceroses of all species has been questioned. The development of reproductive pathology in captive female rhinoceroses of all species has been fairly well documented (Godfrey et al., 1991; Schaffer et al., 1994; Hermes et al., 2004; Hermes et al., 2014), but data on male reproductive pathology and associated infertility is limited. One report of fertility assessments on six male rhinoceroses of four species, revealed a rather high incidence of abnormal findings (Hermes et al., 2006) suggesting male rhinoceros fertility problems are not uncommon. Although our analysis of male rhinoceroses in North American zoos did confirm the occurrence of gross testicular pathology, the incidence was relatively low and the subsequent impact on sperm production and quality was negligible in most cases with just one rhinoceros (4% of total) azoospermic, and thus, unquestionably infertile.

Previous research has shown for all mammalian species examined that rapid removal of testicular tissue and epididymides and their storage at cooler temperatures until sperm is harvested results in higher quality sperm samples (Kikuchi et al., 1998; An et al., 1999; Lone et al., 2011; Yu and Leibo, 2002; Martinez-Pastor et al., 2005; Soler et al., 2005; Fernández-Santos et al., 2011; Monteiro et al., 2013). Case reports in the rhinoceros also have demonstrated that quality sperm samples can be obtained post-mortem if reproductive tissue is removed rapidly and stored cool until harvest (Williams et al., 1995; Lubbe et al., 1999; O'Brien and Roth, 2000). Data from this analysis are in agreement with these previous reports and further support the recommendation that testicular tissue be removed rapidly and cooled after a rhino's death to improve the odds of rescuing quality sperm samples. The relatively high proportion of harvest attempts (70%) from rapidly removed and cooled tissues yielding quality sperm samples was encouraging. There were relatively few sperm rescue attempts from tissues that were not rapidly removed and cooled from rhinoceroses post-mortem and most failed to produce quality samples. However, in one case, tissues recovered from an animal found dead in the morning of natural causes did yield a quality sample. It is possible the animal died within 1–2 h of the staff's arrival so tissues were harvested rapidly post-mortem but it may also be possible to obtain quality samples from testicular tissue removed more than 4 h post-mortem, and such attempts should not yet be abandoned. In conjunction with IVF, live offspring have been produced with sperm recovered from mice stored at room temperature (~22 °C) for up to 24 h even though sperm motility in samples recovered >12 h post-mortem had declined significantly (Songsassen et al., 1998). The environmental conditions, specifically temperatures, at the time of the rhino's death could be a significant factor in these cases of delayed testicular removal. Sperm samples containing on average 66% motile sperm have been recovered from epididymides harvested over 12 h post-mortem in the Spanish ibex in an environment with temperatures at 12 °C (Fernández-Santos et al., 2011).

Although no obvious effect on sample quality related to the time interval from death to sperm recovery was noted

in this study, the longest time recorded was just 51 h and the sample collected did meet the cryopreservation criteria. It is likely that other factors including rapid removal and cooling are more important in affecting sample quality and overshadow any impact storage time may have on sample quality during the relatively short time frame covered in this analysis. In horses, quality samples that retain viability and fertilizability can be harvested up to 96 h post-mortem as long as tissues are removed rapidly and stored at 4 °C (Vieira et al., 2013). Similar results of have been achieved with other species (Kikuchi et al., 1998; An et al., 1999; Kishikawa et al., 1999; Yu and Leibo, 2002; Soler et al., 2005).

In contrast to post-mortem sperm harvest studies in domestic and hunted species, such efforts in endangered species are potentially impacted by several additional factors. Because post-mortem sperm harvest attempts in endangered species are likely to be performed on aged and/or ailing individuals and not individuals in the prime of their life, it was encouraging to note that sperm samples could be harvested from tissues of older animals including the oldest animal in the study (43 year), indicating that rescue attempts should be performed on rhinoceroses regardless of age. Furthermore, quality samples could be recovered from rhinoceroses that had suffered prolonged illness such as renal disease and hemochromatosis. Finally, we questioned if euthanasia by iv injection of Euthasol could have a detrimental effect on sperm cell viability and motility. In contrast, our data actually suggested a benefit (though not statistically significant) to euthanasia over natural death with regard to our ability to harvest quality sperm samples. However, this result is tightly confounded with time of tissue removal and cooling since tissues from euthanized animals were likely to be processed efficiently, whereas unexpected deaths were more likely to be associated with delayed tissue removal and cooling. Regardless, there did not appear to be any negative impact of euthanasia on sperm sample quality.

Although seasonal impacts on post-mortem sperm quality have been reported in Iberian red deer (Martinez-Pastor et al., 2005), the species experiences significant reproductive seasonality that explains the seasonal effect. It is likely that many seasonally reproductive endangered species would be similarly impacted. Rhinoceroses are not seasonally restricted in reproductive activity and no seasonal impact on their sperm quality was anticipated. However, the concern regarding environmental factors during different seasons and how those factors might impact tissue recovery, processing and shipping were of interest in this study. Clearly, results indicated that quality sperm samples could be recovered from rhinoceros tissues during any season of the year, but it was interesting to note that the five tissue samples shipped in January, July and August did not yield quality samples. These months represent one of the two coldest and the two hottest months of the year, either of which could have impacted tissue quality in vivo immediately post-mortem and/or conditions during shipping regardless of careful preparation by the sending facility.

Perhaps the most interesting finding of this study was unexpected and not associated with the primary goals or hypotheses of this analysis. The total number of sperm harvested from the Indian rhinoceros compared to that for all other rhinoceros species was noteworthy. On average, the Indian rhinoceros appears to be producing 10× more sperm than the other species. This difference could not be explained by the age of the species because the median age of the Indian rhinoceroses (32 year) was the highest and that for the African black rhinoceroses and African white rhinoceroses was about the same (21 year and 20 year, respectively). Although the sample size of Indian rhinoceroses was relatively small, the difference in sperm production between this species and the other rhinoceros species can also be ascertained from previous publications (Hermes et al., 2005; Roth et al., 2005; Roth, 2006; Stoops et al., 2010). However, in most of those studies electroejaculated semen samples were the source of sperm so total sperm collected was considered a product of the procedure's effectiveness and the effects of the anesthetics used and not necessarily representative of what the male rhinoceroses were producing. Data from this study are the first comparative data that support the theory that Indian rhinoceros bulls may produce more sperm than bulls of the other rhinoceros species. However, our sample sizes for Indian and white rhinoceroses were rather small and more robust numbers would be necessary to make any conclusive statements regarding this potential species' difference.

Although percent sperm motility was variable among samples cryopreserved in this study, the loss of just ~15% of sperm motility on average post-thaw was comparable to what has been reported for rhinoceros sperm collected by electroejaculation or post-coital recovery and cryopreserved by similar methods (O'Brien and Roth, 2000; Stoops et al., 2010). Furthermore, these post-thaw results compare favorably to results reported for sperm harvested post-mortem and cryopreserved in the Spanish ibex (Fernández-Santos et al., 2011) and horse (Monteiro et al., 2013). Although no offspring have been produced yet using post-mortem epididymal rhinoceros sperm samples, one embryo was reported following oocyte IVM and IVF with sperm rescued from a male black rhinoceros 30 h post-mortem and cryopreserved for 6 year (Stoops et al., 2011).

In summary, study results demonstrate the promise of post-mortem sperm rescue and cryopreservation in rhinoceroses maintained in North American zoos. Quality samples can be recovered from rhinoceroses of all mature ages following their death, regardless of the cause. Prompt tissue recovery, cooling and proper packaging/shipping are the most important factors affecting the ability to recover quality samples. The previously reported method of rhinoceros semen cryopreservation is equally effective with epididymal sperm, and post-thaw samples appear to maintain the functional capacity to produce embryos via IVF or AI. Given the tenuous existence of all rhinoceros species, sperm rescue should become standard practice for all North American zoos maintaining this taxon within their collections to preserve extant

genetic diversity and expand future options for species management.

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