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Ejaculate Traits and Sperm Cryopreservation in the Endangered Baird's Tapir (*Tapirus bairdii*)

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ABSTRACT: There is little information on the reproductive biology of the male Baird's tapir (*Tapirus bairdii*). In this study, we characterized the ejaculate traits and evaluated the efficacy of 2 cryodiluents on sperm cryosurvival. Ejaculates were assessed for volume, pH, sperm motility, forward progression, osmolality, sperm concentration, sperm morphology, and acrosomal integrity. For cryopreservation, ejaculates with >50% total sperm motility were washed, and sperm pellets were resuspended in either Botu-Crio (CryoVital, Grandau, Germany) or INRA 96 containing 2% egg yolk and 2.5% each of methyl- and dimethylformamide (INRA 96), and they were cryopreserved over liquid nitrogen vapor. Thawed samples were incubated in vitro (25°C) and evaluated for percent total sperm motility, forward progression, and acrosomal integrity at hourly intervals for 4 hours. Spermic ejaculates were obtained from all males, and the mean seminal volume, sperm concentration per milliliter, percent sperm motility, progressive status, and percent morphologically normal cells were 20.4 ± 4.3 mL, 101.2 ± 24.0 × 10^6/mL, 46.1% ± 5.0%, 2.9 ± 0.1, and 6.9% ± 1.4%, respectively. There was a positive significant correlation between percent normal sperm and animal age ($r = 0.66$; $P < .004$). Cryopreservation in either Botu-Crio or INRA 96 resulted in a decline ($P < .05$) in percent sperm motility and acrosomal integrity. Sperm forward progression remained unaffected immediately after thawing in INRA 96 but continued to decline over time. These results characterize, for the first time, the ejaculate traits of the tapir; demonstrate that tapir spermatozoa can be cryopreserved in diluents containing amides alone or in combination with glycerol; and provide fundamental information critical for development of assisted reproductive technologies for the Baird's tapir.

Key words: Tapirs, electroejaculation, amides.

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Tapirs belong to the family Tapiridae and comprise 4 distinct species with different geographical distribution. They include the lowland tapir (*Tapirus terrestris*), Baird's tapir (*Tapirus bairdii*), mountain tapir (*Tapirus pinchaque*), and Malayan tapir (*Tapirus indicus*). The Baird's tapir is listed as endangered by the International Union for Conservation of Nature and Natural Resources Red List because of low numbers in the wild (2010). Baird's tapir are endemic to Belize, Colombia, Costa Rica, Guatemala, Honduras, Mexico, Nicaragua, and Panama. For decades, considerable work has focused on the ecology and in situ conservation of this species (Foerster and Vaughan, 2002; Castellanos et al, 2008; Naranjo, 2009) combined with an ex situ conservation program that is based in zoological institutions throughout the world (Barongi, 1993). Major objectives of the ex situ program are to establish insurance populations as a hedge against extinction and retain 90% of the genetic variability existing at the beginning of the captive breeding program over a period of 100 years. The current global ex situ population is comprised of 120 animals distributed among 46 institutions and could benefit from integration of additional founder animals (Roman, 2009).

Baird's tapirs continue to be persecuted in many communities within their native range for supplemental food. Furthermore, their native habit continues to decline because of changes in land use practices (Castellanos et al, 2008). Despite these increased threats, little effort has been directed at understanding their population status within range countries. In Panama, Baird's tapir is listed as endangered and protected by law, and greater than 25 animals distributed among 3

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institutions are maintained in captivity. However, a large proportion of this captive population is threatened with increased inbreeding as a result of outdated management practices and inadequate pairing and mate selection choices.

Conservation of the Baird's tapir may benefit from a variety of assisted reproductive technologies, including collection, preservation and use of spermatozoa, artificial insemination, in vitro embryo production, embryo transfer, and preservation of gonadal tissues (Wildt, 1990; Leibo and Songsasen, 2002; Pukazhenthi and Wildt, 2004; Pukazhenthi et al, 2006; O'Brien and Robeck, 2010). The value of these technologies has been demonstrated in a variety of species (Pukazhenthi and Wildt, 2004; Pope et al, 2006; Swanson, 2006; Hermes et al, 2007; O'Brien et al, 2009) via production of live offspring. In felids and canids, tremendous advances have been facilitated by detailed investigations conducted on domestic animal models, including the cat and dog, respectively (Luvoni, 2000; Brown, 2006; Comizzoli et al, 2009; Thomassen and Farstad, 2009). Despite the evolutionary relationship to equids and rhinoceros, tapirs belong to a unique genus and family within the order Perissodactyla, and no suitable domestic animal models exist (Norton and Ashley, 2000). Hence, the reproductive biology of the Baird's tapir remains to be elucidated. Our current understanding of this species is based largely on direct observations and studies focused on the endocrinology of the female Baird's (Brown et al, 1994) and Malayan tapir (Kusuda et al, 2007). Briefly, Baird's tapir breed throughout the year in both hemispheres and typically produce a single offspring after 13-month gestation. Males reach sexual maturity before they are 3 years old, and females are sexually mature around 2 years of age. They have a long lifespan, and males older than 20 years are able to sire live young.

Knowledge of the reproductive characteristics of a species is important for developing reproductive technologies aimed at their long-term preservation (Pukazhenthi and Wildt, 2004). There have been numerous studies on the collection, characterization, and/or cryopreservation of spermatozoa in a host of domestic and nondomestic ungulate species (King and Macpherson, 1973; Howard et al, 1981; Love, 1992; Monfort et al, 1993; Cassinello et al, 1998; Adams et al, 2009). Collection of viable spermatozoa has been achieved using an artificial vagina (King and Macpherson, 1973; Love, 1992), electroejaculation (Howard et al, 1981; Howard et al, 1984; Monfort et al, 1993; Cassinello et al, 1998; Wirtu et al, 2008; Saragusty et al, 2010), or rectal massage technique (Schmitt and Hildebrandt, 1998), or through operant conditioning using positive-reinforcement schedule (O'Brien and Robeck, 2010). However, there are no reports of successful semen collection in the tapir.

Tapirs are evolutionarily related to equids and the rhinoceros (Norton and Ashley, 2000). Although domestic stallions can be routinely collected using an artificial vagina, this approach is not applicable for nondomestic species, including tapirs, because of imminent risk of injury to individuals attempting the collection. Recently, electroejaculation has yielded encouraging results in both the domestic stallion (Cary et al, 2004) and the critically endangered Przewalski's horse (Collins et al, 2006). Likewise, this technique also has been developed for the rhinoceros, resulting in collection of good-quality ejaculates (Hermes et al, 2005; Roth et al, 2005; Hermes et al, 2007).

Spermatozoa have been successfully cryopreserved in numerous nondomestic species (Monfort et al, 1993; Saragusty et al, 2006; Hermes et al, 2007; Garde et al, 2008; Hermes et al, 2009; Stoops et al, 2010). Interestingly, most cryodiluents are egg yolk or skim-milk based, contain glycerol or dimethylsulfoxide, and have yielded variable results. Recent studies have examined alternative cryoprotectants, including amides, for cryopreserving spermatozoa with a high degree of success (Squires et al, 2004; Alvaranga et al, 2005; Bianchi et al, 2008; Zee et al, 2008). On the basis of the evolutionary relationship of tapirs to equids, we were interested in comparing 2 cryodiluents (Botu-Crio and INRA 96 supplemented with amides) used in cryopreservation of stallion spermatozoa (Alvaranga et al, 2005; Carmo et al, 2005). Specifically, whereas Botu-Crio consisted of a base medium supplemented with 20% egg yolk, 1% glycerol, and 4% methylformamide, INRA 96 (IMV Technologies, Maple Grove, Minnesota) consisted of skim milk (as base medium) supplemented with 2% egg yolk and 2.5% each of methyl- and dimethylformamide. Therefore, the aims of the present study were to characterize Baird's tapir ejaculate traits and to examine the ability of tapir spermatozoa to survive cryopreservation in various cryodiluents.

Materials and Methods

Animals

Baird's tapir (n = 11 males; ages, 2–21 years; estimated body weight, 280–300 kg) were housed in 3 institutions in the Republic of Panama. Two institutions, Summit Park Zoo and Botanical Gardens (SPZ; n = 3 males; n = 1 proven breeder) in Panama City and El Nispero Zoo (n = 1 male; proven breeder) were zoological organizations with public access. The third institution, Villa Griselda (n = 7 males; n = 5 proven breeders) located in El Valle de Anton was a private collection that was closed to the public. Males were housed alone (Summit Park) or with a female (El Nispero) or with 1 or more females (Villa Griselda). Animals were fed a variety of diets, including a commercial equine pelleted diet supplemented with

fresh vegetables and fruits (SPZ); corn meal supplemented with produce (fresh greens, tubers, and fruits) and fresh browse (El Nispero Zoo); or a commercial equine pelleted diet supplemented with tapioca, fresh vegetables, and fruits (Villa Griselda). All animals had access to water ad libitum and shelter. All animal procedures were reviewed and approved by the Smithsonian Conservation Biology Institute Animal Care and Use Committee.

Anesthesia, Semen Collection, and Evaluation

Male tapirs were anesthetized in 2007 (April) and 2008 (January and October) on the basis of estimated weights, using a combination of detomidine hydrochloride (0.06 mg/kg; Dormosedan; Pfizer Animal Health, Exton, Pennsylvania) and butorphanol tartrate (0.15–0.20 mg/kg; Equanol; Vedco Inc, St Joseph, Missouri) with ketamine hydrochloride (1–2mg/kg; Vedco Inc) given either in conjunction or as a supplement to reach a plane of recumbency and light anesthesia. Anesthesia was maintained using boluses of propofol (0.2–2.0 mg/kg per bolus, administered slowly to effect at an average rate of 0.029 mg/kg/min; PropoFlo; Abbott Animal Health, Abbott Park, Illinois). Heart rate, respiration, oxygen saturation, and tidal carbon dioxide were monitored throughout the procedure. The rectum was evacuated using a well-lubricated gloved hand, and the penis was carefully exteriorized and washed thoroughly with sterile saline and wiped dry before attempting semen collection.

Prior to the start of electroejaculation, testes measurements were recorded using calipers (length and width) and a real-time hand-held ultrasound with a 5-MHz transducer (to measure testicular height). Electroejaculation was carried out following a modified procedure described by Howard (1993). A rectal probe (5.2-cm diameter) with 3 longitudinal electrodes was inserted approximately 6 inches into the rectum and was connected to a 60-Hz sine wave electrostimulator (PT Electronics, Boring, Oregon). Electrical stimuli were delivered starting at 2 V (2–5 stimulations) and gradually increased to 3 V (2–5 stimulations), 4 V (10–20 stimulations), 5 V (10–20 stimulations), and 6 V (10–20 stimulations). The position of the rectal probe and voltage applied was primarily determined by the successful collection of spermic ejaculates. Each series was comprised of 30 to 40 stimulations, and a minimum of 3 series was applied to all males. Ejaculates were collected into sterile polypropylene specimen cups (8 oz) and maintained at ambient temperature (28°C to 30°C) until evaluation.

At the end of each series, ejaculates were evaluated for volume, pH (EMD colorpHast pH strips; Thermo Fisher Scientific, Waltham, Massachusetts), osmolality (Wescor Inc, Logan, Utah), sperm percent total motility, and forward progressive status (scale = 0–5, with a rating of 5 equivalent to rapid, straightforward progress [Howard, 1993]) using a phase contrast microscope at ×200 magnification. Sperm concentration was determined using a hemocytometer. Ejaculate from each cup was transferred into a sterile 15-mL or 50-mL conical centrifuge tube, volume was recorded, and ejaculate was slowly diluted (1:3) with warm INRA 96 and maintained at ambient temperature protected from light. An aliquot (20 μL) was fixed in 0.1 mL of 0.3% glutaraldehyde in PBS (pH 7.4; 340 mOsm) for assessment of sperm morphology (Figure 1). Spermatozoa were classified as normal or as having one of the following abnormalities: 1) head abnormalities, including microcephalic, macrocephalic and bi- or tricephalic; 2) acrosomal abnormalities, including missing or damaged acrosomal membranes; 3) midpiece abnormalities, including abnormal or missing midpiece, a bent midpiece with retained cytoplasmic droplet, and a bent midpiece with no droplet; 4) tail abnormalities, including tightly coiled tail, bent tail with retained cytoplasmic droplet, bent tail with no droplet, bi- or triflagellate, retained proximal droplet, and retained distal droplet; and 5) other abnormalities, including spermatid and bent neck. Another aliquot (50 μL) was fixed in 1 mL of 4% paraformaldehyde for assessing acrosomal status, as described below. Upon completion of the collection procedure, ejaculates with similar quality (motility and forward progression score) were pooled, divided into 2 aliquots, and centrifuged (500 × *g*; 15 minutes), and supernatant was discarded.

Testicular volume and estimated daily sperm output was calculated as previously described (Love, 1992). Briefly, testicular volume was estimated using the following formula: volume = 0.5233 × length × width × height. Daily sperm output (DSO; 10^9/day) was calculated using the formula DSO = (0.024 × total testicular volume) – 1.26, in which total testicular volume represents the sum of right and left testis volume.

Sperm Cryopreservation, Thawing, and Evaluation

For sperm cryopreservation, sperm pellets (n = 4 males; 1 ejaculate per male) were resuspended in either Botu-Crio or INRA 96 containing 2% (v/v) egg yolk, 2.5% (v/v) methylformamide and 2.5% (v/v) dimethylformamide and processed for cryopreservation. Botu-Crio contained a base medium and 20% (v/v) egg yolk, 1% (v/v) glycerol and 4% (v/v) methylformamide. Sperm suspensions (250×10^6 motile sperm/mL) were packaged into 0.5-mL plastic straws, heat sealed and cooled for 20 minutes at 5°C. Straws were then cryopreserved over liquid nitrogen vapor (4 cm above vapor; 15 minutes) and plunged into liquid nitrogen. Cryopreserved samples were transported to the Smithsonian Conservation Biology Institute in Washington, DC, for analyses.

Straws were thawed at 46°C for 20 seconds, and contents were emptied into a sterile eppendorf tube and maintained at ambient temperature (25°C) and protected from light. On the basis of preliminary studies, we had determined that dilution of thawed samples in INRA 96 or Hepes-buffered Ham's F10 was detrimental to sperm motility (data not shown). For assessment of sperm longevity, sperm motility and progressive status were recorded immediately after thawing (postthaw) and at hourly intervals for 4 hours. At each assessment interval, an aliquot (20 μL) was fixed in 4% paraformaldehyde and was later assessed for acrosomal integrity, as described shortly.

Evaluation of Acrosomal Integrity

Sperm samples fixed in 4% paraformaldehyde were centrifuged for 8 minutes at 2000 × *g*, and the supernatant was discarded. Pellets were washed twice with 500 μL of 0.1 M ammonium

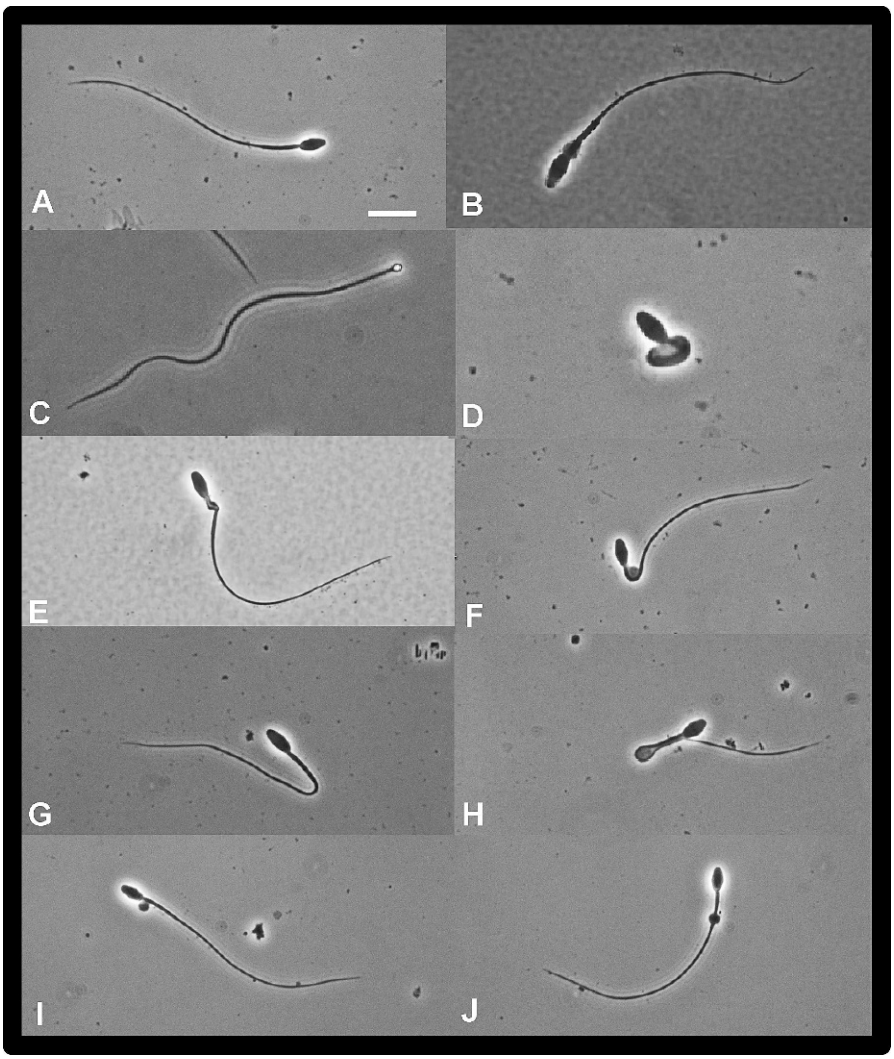

Figure 1. Phase-contrast photomicrographs of normal and abnormal spermatozoa commonly observed in Baird's tapir electroejaculates (×1000). **(A)** normal; **(B)** abnormal acrosome with proximal cytoplasmic droplet; **(C)** microcephalic; **(D)** tightly coiled flagellum; **(E)** abnormal midpiece; **(F)** bent midpiece with cytoplasmic droplet; **(G)** bent flagellum without cytoplasmic droplet; **(H)** bent flagellum with cytoplasmic droplet; **(I)** proximal cytoplasmic droplet; **(J)** distal cytoplasmic droplet. Scale bar = 10 μm.

acetate (pH 9.0), and the pellet was resuspended in approximately 50 μL of the ammonium acetate solution. An aliquot of this suspension was smeared onto microscope slides and allowed to dry at ambient temperature. Thereafter, slides were flooded with 0.22% Coomassie stain (Coomassie Blue G-250; Thermo Fisher Scientific, Waltham, Massachusetts) in 50% methanol, 10% glacial acetic acid, and 40% deionized water (Larson and Miller, 1999) for 90 seconds, rinsed with deionized water, dried at ambient temperature, and permanently preserved by placing a coverslip over a drop of mounting medium (Krystalon; EM Science, Gibbstown, New Jersey). For each sample, 200 spermatozoa were assessed individually for acrosomal integrity using bright field microscopy at ×1000 and categorized as intact, damaged, or nonintact (Figure 2). Briefly, tapir spermatozoa with intact acrosomal membranes exhibited a uniform blue staining overlying the acrosomal region. Spermatozoa with nonintact or damaged acrosomes displayed a clear area overlying the acrosomal region or a patchy staining pattern.

Statistical Analyses

Data were analyzed using SAS (SAS Inc, Cary, North Carolina). Semen characteristics are presented as mean ± standard error of the mean (SEM). Data were checked for normality by Kolmogorov-Smirnov test. Data for volume, sperm motility, normal morphology, and acrosomal integrity were compared among individual animals using Tukey's test. Data for postthaw sperm motility and acrosomal integrity were ArcSin transformed and compared by Tukey's test. Relationship between animal age and percentage of normal sperm was analyzed using Pearson correlation. Results were considered statistically significant when $P < .05$.

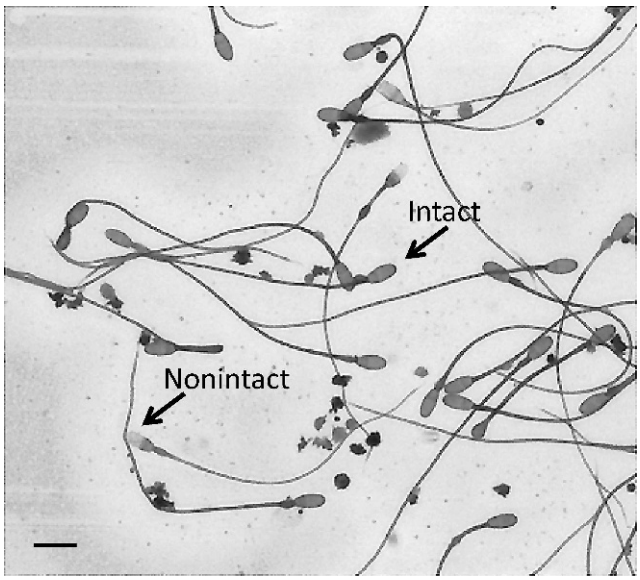

Figure 2. Coomassie blue staining of Baird's tapir spermatozoa for assessment of acrosomal integrity (×1000). Spermatozoa with intact acrosomes exhibited a uniform purple staining overlying the acrosomal region. Spermatozoa with nonintact acrosomes displayed a clear or patchy staining pattern. Scale bar = 10 μm.

Results

Testicular Measurement and Semen Parameters in Male Baird's Tapir

Testes in tapirs were located ventral to the external anal sphincter, elliptical in shape, and enclosed within a tight scrotal sac. Testicular dimensions are summarized in Table 1. Mean testicular volumes (± SEM) for the left and right testes were 129.9 ± 8.9 cm^3 and 132.9 ± 8.1 cm^3, respectively, with an estimated daily sperm output of 5.04 ± 0.4 × 10^9 cells. All males produced spermic ejaculates, but ejaculate quality varied among individuals (Table 2), resulting in a high degree of variation among males. Poor quality ejaculates were routinely comprised of low volume, low sperm concentration, and low percent sperm motility. Ejaculates were milky white in appearance. The osmolality of semen ranged from 203 to 413 mOsm, with an average of approximately 284 mOsm, and the pH was slightly alkaline (7.4 ± 0.1; range, 6.5–8.5).

The mean seminal volume, sperm concentration per milliliter, percent sperm motility, progressive status, and percent morphologically normal cells were 20.4 ± 4.3 mL, 101.2 ± 24.0 × 10^6/mL, 46.1 ± 5.0, 2.9 ± 0.1, and 6.9 ± 1.4, respectively (Table 2). All ejaculates contained a high proportion of morphologically abnormal sperm (Table 3). Predominant morphological defects in freshly collected ejaculates were spermatozoa with abnormal acrosomes (approximately 34%) and spermatozoa with retained proximal cytoplasmic droplet (approximately 28%). Despite these abnormalities, more than 74% of spermatozoa exhibited intact acrosomal membranes as assessed by the Coomassie blue staining method.

Table 1. *Testicular measurements in the Baird's tapir*[a]

Measurement	Left	Right
Length (cm)	10.6 ± 0.2 (8.7–12.0)	10.5 ± 0.2 (9.3–12.3)
Width (cm)	4.7 ± 0.1 (3.7–5.5)	4.9 ± 0.2 (4.0–5.7)
Height (cm)	4.9 ± 0.2 (3.3–6.2)	4.8 ± 0.1 (3.9–5.6)
Volume (cm^3)	129.9 ± 8.9 (55.9–180)	132.9 ± 8.1 (80.3–179.2)

[a] Values represent mean ± standard error of the mean (n = 10 males); values within parentheses represent minimum and maximum.

Tapir spermatozoa in raw ejaculates when cooled to ambient temperature rapidly lost progressive motility, and the majority of spermatozoa exhibited a whiplash-like motility. However, ejaculates diluted in INRA 96 maintained higher percent sperm motility and forward progression both at ambient temperature and at 37°C (on a heated microscope stage; data not shown). Centrifugation at 500 × *g* (15 minutes) resulted in approximately 15% decline in total sperm motility in tapir ejaculates.

Correlation Between Donor Age and Semen Traits

Volume and sperm concentration were lower in 2-year old males (data not shown). In contrast, males 3 years and older consistently produced large volume ejaculates with higher sperm concentration and percent sperm motility. The oldest male evaluated in this study was 21-years-old and consistently produced good quality ejaculates. There was a positive significant relationship between the age of animal and the percentage of morphologically normal spermatozoa ($P < .004$). No significant positive relationships ($P > .05$) were detected between donor age and ejaculate volume, sperm concentration per milliliter, total sperm, or percent sperm motility.

Sperm Cryopreservation

Semen traits of ejaculates used for cryopreservation are summarized in Table 4. Although there were no differences in most semen traits evaluated, percent sperm motility was higher ($P < 0.05$) in ejaculates used for cryopreservation (66.2% ± 7.5%) compared with all ejaculates combined (46.1% ± 5.0%).

Percent sperm motility declined ($P < .05$) immediately after thawing in both cryodiluents (Figure 3A). Although no further decline was observed in aliquots cryopreserved in Botu-Crio, samples cryopreserved in INRA 96 declined further at 3 hours and 4 hours compared with motility immediately after thawing and

Table 2. *Ejaculate characteristics of captive Baird's tapir in Panama*[a]

Trait	Mean ± SEM	Minimum	Maximum	No. of Ejaculates
Seminal volume (mL)	20.4 ± 4.3	0.5	65.0	23
Sperm concentration (×106/mL)	101.2 ± 24.0	1.5	430.0	23
Sperm motility (%)	46.1 ± 5.0	10.0	80.0	23
Sperm forward progressive status[b]	2.9 ± 0.1	2.0	3.5	23
Morphologically normal sperm (%)	6.9 ± 1.4	1.0	24.0	16
Intact acrosomes (%)	74.8 ± 3.8	50.5	98.5	16
Osmolality (mOSm)	284.7 ± 14.5	203.0	413.0	12
pH	7.4 ± 0.1	6.5	8.3	22

Abbreviation: SEM, standard error of the mean.

[a] n = 11 males.

[b] Forward progressive status scale = 0–5, in which 5 is the most rapid, straightforward progression.

1 hour after in vitro incubation. There was no difference ($P > .05$) in percent sperm motility between Botu-Crio and INRA 96 until 3 hours after incubation. At the end of 4 hours at 25°C, spermatozoa cryopreserved in Botu-Crio maintained higher ($P < .05$) percent sperm motility compared with aliquots cryopreserved in INRA 96 (Figure 3A).

Sperm forward progression after thawing remained unaffected ($P > .05$) in aliquots cryopreserved in INRA 96 but declined sharply ($P < .05$) in Botu-Crio (Figure 3B). In vitro incubation resulted in no further decline ($P > .05$) in progressive motility in aliquots cryopreserved in Botu-Crio. In contrast, spermatozoa cryopreserved in INRA 96 exhibited a further decline ($P < .05$) in progressive motility at 2 hours compared with raw ejaculate or immediately after thawing. Progressive motility also was higher ($P < .05$) in aliquots cryopreserved in INRA 96 at 1 hour and 2 hours of incubation at 25°C compared with aliquots cryopreserved in Botu-Crio (Figure 3B).

The percentage of spermatozoa with intact acrosomal membranes declined sharply ($P < .05$) immediately after thawing in both Botu-Crio (approximately 49%) and INRA 96 (approximately 43%; Figure 3C). There was no further reduction in acrosomal integrity following in vitro incubation at 25°C in either cryodiluent. Furthermore, no differences were noted in the percentage of spermatozoa with intact acrosomal membranes between Botu-Crio and INRA 96.

Table 3. *Average percentage (± SEM) of normally and abnormally shaped spermatozoa in electroejaculates from captive Baird's tapir in Panama*[a]

Trait	Mean ± SEM	Range
Normal sperm	6.9 ± 1.4	1–24
Abnormal sperm	93.1 ± 1.4	76–99
Macrocephalic	0.1 ± 0.1	0–2
Microcephalic	0.1 ± 0.1	0–2
Bicephalic	0.3 ± 0.1	0–1
Abnormal acrosome	34.3 ± 3.9	13–65
Abnormal midpiece	0.3 ± 0.1	0–2
Tightly coiled flagellum	4.0 ± 1.5	0–27
Bent midpiece with droplet	14.0 ± 2.9	0–50
Bent midpiece without droplet	2.6 ± 0.7	0–12
Bent flagellum with droplet	0.8 ± 0.5	0–9
Bent flagellum without droplet	0.3 ± 0.1	0–2
Proximal droplet	28.6 ± 4.6	6–67
Distal droplet	5.6 ± 1.7	0–31
Bent neck	0.2 ± 0.1	0–2
Spermatid	1.4 ± 0.4	0–6
Detached head	0.1 ± 0.1	0–2

Abbreviation: SEM, standard error of the mean.

[a] n = 11 males, n = 18 ejaculates.

Discussion

The present study is the first systematic effort to characterize the ejaculate traits of the endangered Baird's tapir and to develop effective field-friendly sperm cryopreservation techniques. We demonstrate that electroejaculation is a safe and efficient method for semen collection in anesthetized Baird's tapirs and that tapir spermatozoa can be cryopreserved under field conditions using a commercial cryodiluent (Botu-Crio) or a cryodiluent comprising of INRA 96 supplemented with 2% egg yolk and 2.5% each of methyl- and dimethylformamide. These results represent the start of a database for the Tapiridae family comprised of 4 different species, all of which are listed as vulnerable or endangered with extinction.

The efficiency of obtaining spermic ejaculates was 100%, but the ejaculate quality was highly variable. Although no information is available on tapirs, our data were comparable to previous reports on domestic stallions (Cary et al, 2004), the Przewalski's horse (Collins et al, 2006), and the rhinoceros (Hermes et al, 2005; Roth et al, 2005; Stoops et al, 2010), species taxonomically related to the tapir. Interestingly, unlike the horse and rhinoceros, Baird's tapir electroejaculates were not comprised of pre-ejaculate and sperm-rich

Table 4. *Characteristics of ejaculates used for cryopreservation*[a]

Trait	Mean ± SEM
Seminal volume (mL)	23.2 ± 12.1
Sperm concentration ($\times 10^6$/mL)	180.7 ± 56.1
Sperm motility (%)	66.2 ± 7.5
Sperm forward progressive status[b]	3.5 ± 0.2
Morphologically normal sperm (%)	9.6 ± 2.1
Intact acrosomes (%)	80.6 ± 1.8
Osmolality (mOSm)	282.2 ± 5.2
pH	7.1 ± 0.3

Abbreviation: SEM, standard error of the mean.

[a] n = 4 males; 1 ejaculate per male.

[b] Forward progressive status scale = 0–5, in which 5 is the most rapid, straightforward progression.

fractions, facilitating the processing of the entire ejaculate.

Electroejaculation in Baird's tapir resulted in ejaculates of large volume and high sperm concentration with moderate motility. Estimated daily sperm output is considered a reliable predictor of testicular function in equids (Love et al, 1992). In the present study, none of the males met the calculated estimate. We predict that sperm output would improve after further refinement of the anesthesia and electroejaculation regimen. The osmolality of semen was similar to that of the domestic horse (Love, 1992), the Przewalski's horse (Collins et al, 2006), and the rhinoceros (Roth et al, 2005; Stoops et al, 2010). Average pH of semen samples in this study was lower than those reported for the domestic stallion, Przewalski's horse (Collins et al, 2006), and the Indian rhinoceros (Stoops et al, 2010) and may be attributed to variations in contributions of the accessory glands in the tapir. The depth of anesthesia, as well as direct effects of the anesthetic drugs used, also may have compounded individual variation between tapirs.

There also were differences in sperm volume among individuals, which may have been due to differences in the total number of stimulations and voltage applied to individual animals. Age has been shown to influence ejaculate volume in several species, including the ram (Rege et al, 2000), bull (Fuerst-Waltl et al, 2006), boar (Smital, 2009), and horse (Dowsett and Knott, 1996). However, we found no significant relationship between ejaculate volume and donor age in the present study. Interestingly, 2 of the 11 males evaluated in the present study were difficult to anesthetize and maintain at a surgical plane; hence, we were unable to conduct the typical stimulation protocol, which might have contributed to low ejaculate volume and sperm concentration.

Baird's tapir ejaculates consistently contained low numbers of morphologically normal spermatozoa. The most common abnormalities observed in tapir ejaculates

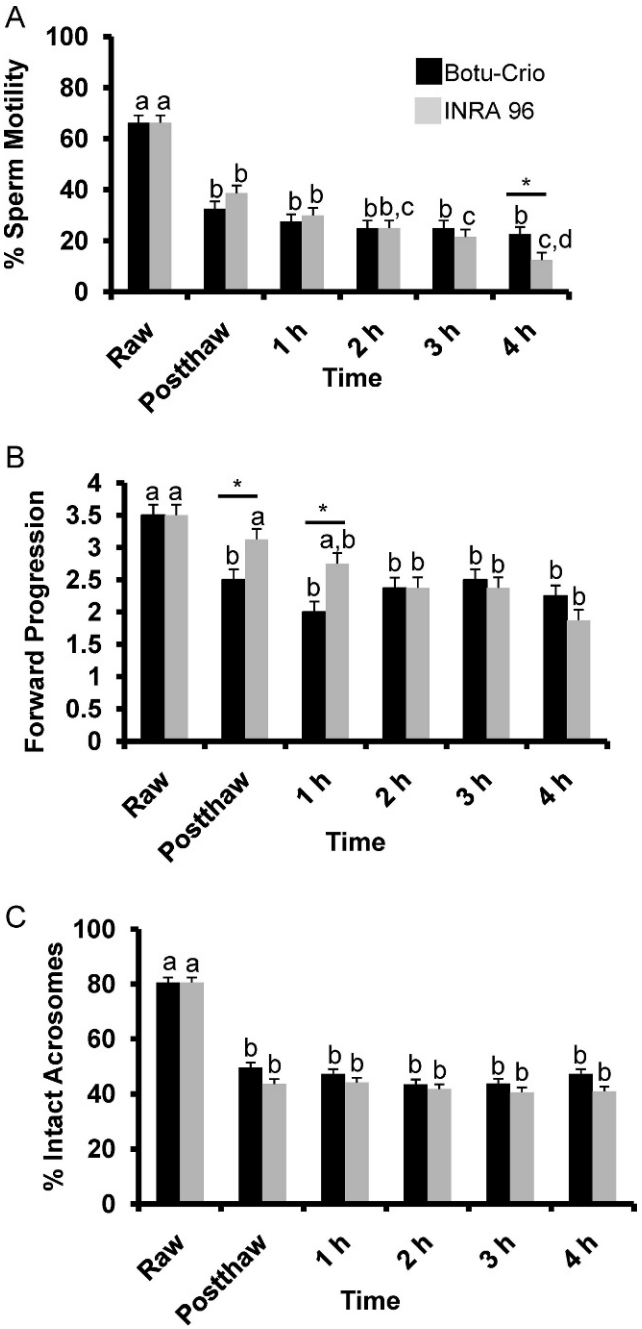

Figure 3. Cryopreservation of Baird's tapir spermatozoa in 2 different cryodiluents, Botu-Crio and INRA 96 supplemented with egg yolk and amides. **(A)** Sperm motility; **(B)** forward progression; **(C)** intact acrosomes. Values are mean ± standard error of the mean. For each cryodiluent, bars with different superscripts indicate significant differences ($P < .05$) among time intervals. Asterisks indicate differences ($P < .05$) between cryodiluents within a time interval.

consisted of spermatozoa with an abnormal acrosome, proximal cytoplasmic droplet, and a bent midpiece with a cytoplasmic droplet. Although acrosomal abnormalities may arise from defects in spermatogenesis, incidence of a high proportion of spermatozoa with a

proximal retained cytoplasmic droplet on ejaculated spermatozoa is generally considered indicative of a defect of testicular origin and has been implicated in the depressed fertility of bulls (Amann et al, 2000; Thundathil et al, 2001) and boars (Kuster et al, 2004). Therefore, additional studies are warranted to examine the etiology of the presence of high proportions of spermatozoa with a proximal cytoplasmic droplet as well as its impact, if any, on fertility of male Baird's tapirs.

Sperm abnormalities have been attributed to age and seasonality and inbreeding status in several species (bull, Soderquist et al, 1996; stallion, Dowsett and Knott, 1996; goat, Karagiannidis et al, 2000; sheep, Rege et al, 2000; deer, Monfort et al, 1993b; antelope, Gomendio et al, 2000). Ejaculates from animals approaching puberty or breeding season also contain high proportions of morphologically abnormal spermatozoa. In the present study, all but one animal was older than 3 years of age. An analysis of the Baird's tapir Studbook indicates that age at first reproduction in captivity is 3 years and older. Furthermore, there is no evidence of seasonality in their breeding behavior (Roman, 2009). Hence, the low proportions of normal sperm in the ejaculate may not be related to animal age or seasonality. Interestingly, some of the males evaluated were a product of incestuous mating (inbreeding), and increased levels of inbreeding has previously been reported to affect ejaculate traits, especially the proportion of morphologically normal sperm in the ejaculate in various felids (Pukazhenthi et al, 2006) and ungulates (Gomendio et al, 2000). However, further genetic analyses of the animals evaluated in this study are warranted to establish the correlation, if any, between the proportion of morphologically normal sperm and inbreeding in this species.

To date, there have been no reports on the cryobiological properties of tapir spermatozoa. In the present study, ejaculates were centrifuged to remove seminal plasma and cryopreserved using a commercial equine semen extender (Botu-Crio) or INRA 96 containing 2% egg yolk and 2.5% each of methyl- and dimethylformamide. Although both cryodiluents induced a decline in the proportion of motile spermatozoa immediately after thawing, 49% and 58% of the initial motility was recovered in Botu-Crio and INRA 96, respectively. Hence, spermatozoa from Baird's tapir appear to be relatively resistant to freeze-thawing induced damage. However, following a 4-hour incubation in the freezing medium, percent sperm motility declined further in both cryodiluents, with 33.9% and 18.8% of the initial motility remaining in Botu-Crio and INRA 96, respectively. These results are similar to previous reports in their closest domestic relative (horse, Wrench et al, 2010) and nondomestic relative (rhinoceros, Hermes et al, 2009; Stoops et al, 2010), wherein freeze-thawing consistently results in an initial decline in sperm motility.

Sperm longevity is influenced by several factors, including media composition (energy substrates), incubation temperature, and cryoprotectant concentration. The rapid decline in tapir sperm motility could have resulted from the toxic effects of glycerol or the amides, because all thawed samples were maintained in the freezing medium at ambient temperature for 4 hours. Preliminary studies in our laboratory demonstrated that dilution of cryoprotectants with TCM199 or Ham's F10 was further detrimental to tapir sperm motility (Della Togna et al, unpublished data). In contrast to sperm percent motility, sperm forward progression remained unaffected until 1 hour after thawing in aliquots cryopreserved in INRA 96 but declined sharply in Botu-Crio during this time period. However, sperm forward progression remained stable in both media until 4 hours of in vitro incubation. Differences in sperm forward progression between the 2 cryodiluents may be attributed to differences in cryodiluent composition. Whereas Botu-Crio consisted of a base medium supplemented with 20% egg yolk, 1% glycerol, and 4% methylformamide, INRA 96 consisted of skim milk (as base medium) supplemented with 2% egg yolk and 2.5% each of methyl- and dimethylformamide. Improved forward progression may have resulted from the lower concentration of egg yolk in INRA 96, which in turn could have made the cryodiluent considerably less viscous. Furthermore, glycerol in Botu-Crio also might have exerted a toxic effect on sperm forward progression. For example, glycerol has been reported to induce cellular damage via protein denaturation, alteration of actin interactions, and induction of protein-free membrane blisters (Alvaranga et al, 2005), all of which could impact sperm survival and motility.

With respect to the acrosomes, both cryopreservation media retained 43% to 49% of spermatozoa with intact acrosomal membranes. This suggests that, similar to sperm motility, freeze-thawing also resulted in extensive damage to Baird's tapir sperm acrosomal membranes. The sharp decline in acrosomal integrity may be attributed to osmotic stress that sperm were exposed to during cryopreservation. Although smaller molecules, such as amides, are believed to permeate the membrane rapidly and minimize osmotic stress, it is likely that both cryodiluents exerted some degree of osmotic stress upon tapir spermatozoa. The benefits of step-wise dilution of glycerol (Wessel et al, 2004) and modifying the sperm membrane via cholesterol preloading on osmotic tolerance of stallion spermatozoa (Glazer et al, 2009) have previously been examined.

Interestingly, although step-wise removal of glycerol was found to mitigate the posthyperosmotic stress characterized by a reduction in both sperm motility and membrane integrity, no benefit was observed in cryopreserved stallion spermatozoa. Likewise, incubation of spermatozoa in cholesterol-loaded cyclodextrin increased the permeability of stallion spermatozoa to cryoprotectants and increased their osmotic tolerance limits (Glazer et al, 2009). Moore et al (2005) also demonstrated that addition of cholesterol to stallion sperm membrane improved sperm cryosurvival. These results clearly demonstrate the need to further examine if either step-wise cryoprotectant removal or cholesterol addition to tapir sperm membranes improves sperm survival. In addition to osmotic stress, reactive oxygen species (ROS) also is known to exert a negative effect on both sperm motility and membrane integrity (Aitken et al, 1997; Ball et al, 2001). Ball et al (2001) characterized the generation of ROS by equine spermatozoa and concluded that damaged and abnormal spermatozoa in ejaculates generate greater amounts of ROS, which could reduce fertility or compromise sperm cryopreservation. There is no information on the endogenous generation of ROS by tapir spermatozoa, but it is possible that ROS production may have influenced survival of cryopreserved tapir spermatozoa and warrants further investigation.

In conclusion, the present study is the first detailed investigation of the basal semen traits and sperm cryopreservation in any tapir species. The results indicate that 1) it is possible to collect and cryopreserve semen from Baird's tapirs managed in captivity and 2) spermatozoa from the Baird's tapir survive cryopreservation when preserved using a protocol developed for use in the horse. Another important by-product of this study was the development of a field-friendly sperm cryopreservation protocol. This new knowledge has immense practical value with respect to genetic management of tapirs maintained in captivity in Panama and elsewhere. Specifically, we now can begin systematically banking sperm from all males maintained in captivity to establish the first ever sperm genome resource bank (Wildt, 1997) for tapirs in the world as well as develop assisted reproductive technologies, such as artificial insemination for this endangered species.

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