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Liquid Semen Storage in Elephants (*Elephas maximus* and *Loxodonta africana*): Species Differences and Storage Optimization

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ABSTRACT: Artificial insemination plays a key role in the genetic management of elephants in zoos. Because freshly extended semen is typically used for artificial insemination in elephants, it has become imperative to optimize conditions for liquid storage and semen transport. The objectives of this study were to examine the interactions between different extenders and storage temperatures on sperm total motility, progressive motility, and acrosomal integrity in Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants. Ejaculates were collected by rectal massage, diluted using a split-sample technique in 5 semen extenders: TL-Hepes (HEP), Modena (MOD), Biladyl (BIL), TEST refrigeration medium (TES), and INRA96 (INR), maintained at 35°C, 22°C, or 4°C. At 0, 4, 6, 12, and 24 hours, aliquots were removed and assessed for sperm total motility, progressive motility, and acrosomal integrity. After 24 hours of storage, African elephant spermatozoa exhibited greater longevity and higher values in sperm quality parameters compared with those of Asian elephants. In both species, semen storage at 35°C resulted in a sharp decline in all sperm quality parameters after 4 hours of storage, whereas storage at 22°C and 4°C facilitated sperm survival. In Asian elephants, MOD and HEP were most detrimental, whereas BIL, TES, and INR maintained motility up to 12 hours when spermatozoa were cooled to 22°C or 4°C. In African elephants, there were no differences among extenders. All media maintained good sperm quality parameters at 22°C or 4°C. However, although MOD, BIL, and INR were most effective at lower temperatures, HEP and TES maintained sperm motility at all storage temperatures. This study demonstrated sperm sensitivity to components of various semen extenders and storage temperatures and offers recommendations for semen extender choices for liquid semen storage for both Asian and African elephants.

Key words: Asian elephant, African elephant, spermatozoa, extender, temperature.

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The North American captive Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant populations are not self-sustaining owing to poor reproduction (Brown, 2000; Olson and Wiese, 2000; Wiese, 2000). Poor reproductive rates are largely due to the limited number of bulls in captivity, few opportunities for natural breeding, high infant mortality, and reproductively older female demographic with many females exhibiting reproductive tract pathologies, ovarian acyclicity, or both that prevent successful conception (Brown, 2000; Olson and Wiese, 2000; Wiese, 2000; Brown et al, 2004b, c). A recent analysis of birth sex ratios in captive elephant populations suggested that a large proportion of artificial insemination (AI) attempts result in male calves (Saragusty et al, 2009a). The reduced fecundity and skewed birth sex ratio further threaten the already unsustainable elephant populations. As a result, the future of North American captive elephant populations has become a major concern for elephant managers throughout the country. In an attempt to increase reproduction, many elephant-holding facilities are now using AI to augment offspring production (Olson and Wiese, 2000; Wiese, 2000; Brown et al, 2004a). Therefore, investigations on semen storage and/or additional semen research, such as sperm sex sorting, are high priorities for developing effective management strategies for elephants.

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Although several studies have explored the efficacy of sperm cryopreservation in Asian (Hedrick and Schmitt, 2001; Thongtip et al, 2004; Saragusty et al, 2009b; Thongtip et al, 2009) and African (Howard et al, 1986; Buice et al, 1995; O'Brien et al, 1997) elephants, postthaw sperm survival remains low and inconsistent. Until reliable sperm cryopreservation technologies are developed for routine use, there is an urgent need to examine short-term (<24 hours) liquid semen storage to minimize loss of sperm viability, motility, and function. Currently, semen is typically collected from multiple bulls housed at different institutions throughout North America. Semen is diluted immediately after collection in a commercial medium, chilled, and shipped to another institution within the country in 24 hours to be used for AI or further processed for cryopreservation and/or sperm sex sorting. Improvements in liquid semen storage within a 24-hour period will not only enhance AI efficacy but also permit routine shipment of samples to centralized laboratories for cryopreservation and additional investigations in semen research, such as sperm sex sorting and understanding mechanisms regulating sperm function.

Although elephants produce large ejaculates, sperm quality is highly variable, and liquid storage in vitro remains a challenge. Elephant semen with or without dilution often exhibits a dramatic decline in sperm motility and viability within 12 hours of storage. The decline in sperm motility and viability may be attributed to rapid depletion of energy substrates as well as the presence of additional factors that inhibit sperm motility (Lindholmer, 1974; Yildiz et al, 2000; Gadea, 2003). Typically when semen is collected from mammalian species and stored, ejaculates are diluted in media that provide spermatozoa with nutrients, prevent microbial growth, and protect cells against damage from cold shock, changes in pH, and osmotic pressure (Gadea, 2003). Many extenders also contain a source of lipoprotein, such as egg yolk or skim milk, to provide additional protection to spermatozoa during cooling. There are various types of media recipes and extender cocktails that are available commercially, and the type of extender that is used for optimal sperm survival may vary according to species and/or the individual (Holt, 2000; Medeiros et al, 2002). Various semen extenders have been examined in an effort to enhance sperm longevity for short- and long-term storage of both African (Jones, 1973; Howard et al, 1986; Buice et al, 1995; O'Brien et al, 1997; Gilmore et al, 1998) and Asian (Kitiyanant et al, 2000; Hedrick and Schmitt, 2001; Graham et al, 2004; Thongtip et al, 2004; Saragusty et al, 2005; Sa-Ardrit et al, 2006; Hermes et al, 2009) elephant spermatozoa. African elephant spermatozoa have exhibited adequate sperm survival in various types of semen extender (Jones, 1973; Howard et al, 1986; Buice et al, 1995; Gilmore et al, 1998), and TL-Hepes extender is currently used for transporting fresh extended semen within a 24-hour period. In contrast, although various types of semen extenders with buffering systems, including Tris–citric acid, TEST, or Hepes, have also been examined in Asian elephants (Hedrick and Schmitt, 2001; Graham et al, 2004; Thongtip et al, 2004; Sa-Ardrit et al, 2006; Portas et al, 2007; Hermes et al, 2009), the survival of Asian elephant spermatozoa during storage appears to be dependent upon an extender with a source of lipoprotein, such as skim milk and egg yolk (Graham et al, 2004; Saragusty et al, 2005; Hermes et al, 2009; Saragusty et al, 2009). Egg yolk–based extenders have been found to provide optimal protection during storage for spermatozoa from many species, including Asian elephants. However, those extenders also interfere with the uniform staining of DNA that is critical to separate X and Y chromosome–bearing populations of sperm during sperm sex sorting (Johnson and Welch, 1999), which is a highly desired goal of elephant conservation efforts. As a result, this study was conducted to identify an extender that will optimize elephant sperm storage within a typical shipment period (≤24 hours) and ideally will be simultaneously compatible with the needs of sperm sex-sorting technology.

In addition to dilution with extender, holding temperature influences how well spermatozoa survive storage. Semen from domestic species is often diluted and cooled to 4°C to reduce the metabolic activity of spermatozoa to enhance sperm survival for longer durations (Blackshaw et al, 1957; Christensen, 1994; Blanchard et al, 1998; Gadea, 2003). Although many of the methods that have been adopted to process elephant semen are based on methods used in domestic species, an extensive and controlled study to specifically examine the optimal holding temperature for storage of elephant spermatozoa has not been conducted. As a result, this study was also designed to determine an optimal holding storage temperature during short-term (≤24 hours) semen storage that will optimize and prolong sperm longevity for both Asian and African elephants.

This is the first comprehensive study to collect semen from multiple captive bulls to systematically investigate and compare semen storage preferences between Asian and African elephants. The specific objectives of this study were to 1) examine the influence of storage media (extenders) and storage temperature on elephant sperm motility and acrosomal integrity, 2) identify an optimal extender and storage temperature that will support sperm survival for up to 24 hours, and 3) determine whether species-specific differences exist in semen

storage conditions between Asian and African elephants.

Materials and Methods

Animals

Semen collections were attempted on 13 Asian (6–35 years; 7 institutions; 131 collections) and 7 African (7–31 years; 4 institutions; 104 collections) elephant bulls in North America. Collection frequency for each bull ranged from 1 to 3 months. All but 3 Asian and 3 African bulls were proven breeders. The bulls were managed under a protected contact training regimen and housed in individual enclosures with visual, olfactory, and/or controlled access to females. The bulls were given free access to water and regular access to feed. Animal research protocols were approved by the Smithsonian Conservation Biology Institute's Institutional Animal Care and Use Committee.

Semen Collection and Evaluation

Semen was collected using the rectal massage technique as previously described by Schmitt and Hildebrandt (1998). Ejaculation was accomplished by massaging the pelvic portion of the rectum dorsal to the urethra and ampullae. Ejaculates were collected into a modified palpation sleeve attached to an insulated 50-mL collection tube to minimize exposure to light and extreme temperature fluctuations. To prevent urine contamination of ejaculates, the collection sleeve was replaced frequently throughout the collection process. The collection tubes containing ejaculates were immediately placed in a portable incubator (Brooder; Dean's Animal Supply, St Cloud, Florida) and maintained at 32°C to 35°C until evaluation. A real-time handheld ultrasound with a 3.5-MHz transducer was used to assess the fullness of the ampullae before and after collection to ensure a complete collection (Schmitt and Hildebrandt, 1998).

Each ejaculate was immediately evaluated for volume (mL), and an aliquot (8 μL) was assessed subjectively for percentage of motile spermatozoa (%tMOT) and forward progressive motility (%pMOT) using a phase-contrast microscope. Sperm concentration was determined using a portable spectrophotometer (DVM rapid test; Value Diagnostics, Spring Valley, Wisconsin) calibrated for measuring concentrations of Asian and African elephant spermatozoa. Osmolality (mOsm) was determined using a vapor pressure osmometer (VAPRO; Wescor Inc, Logan, Utah), and pH was determined using a handheld pH meter (Twin pH; Horiba Ltd, Kyoto, Japan).

Sperm morphology and acrosomal integrity were evaluated using Spermac stain (Conception Technologies, San Diego, California) as described by Baran et al (2004). Briefly, smears were prepared by placing a drop of semen on glass slides, thin smeared, and air dried for 5 minutes. Slides were then fixed in formaldehyde for 5 minutes and rinsed 6 to 7 times in water. Excess water was removed, and the slides were immersed into reagent A for 1 to 2 minutes. This procedure was repeated for reagents B and C per the manufacturer's instructions. The slides were allowed to air dry before assessment. For each sample, a minimum of 200 spermatozoa were assessed individually for acrosomal integrity (%INT) using bright-field microscopy under oil immersion (×1000). Each spermatozoon was categorized as having an intact or nonintact acrosome, and the number of spermatozoa with intact membranes was converted to a percentage (%INT). For morphology, a minimum of 200 spermatozoa were evaluated using phase-contrast optics (×1000).

Preparation of Semen Extenders

Five semen extenders with or without egg yolk were evaluated: 1) Biladyl with 20% egg yolk (BIL; pH 7.0; 320 mOsm; Minitube of America, Verona, Wisconsin); 2) TEST egg yolk refrigeration medium (TES; pH 7.0–7.4; 310–330 mOsm; Irvine Scientific, Santa Ana, California); 3) INRA96 + 4% egg yolk (INR; pH 6.9; 300 mOsm; IMV Technologies, Maple Grove, Minnesota); 4) TL-Hepes (HEP; pH 7.4; 265 mOsm; Cambrex Corp, East Rutherford, New Jersey); and 5) Modena (MOD; pH 6.90; 290 mOsm; SGI International, Cambridge, Iowa). These extenders were chosen to compare efficacy of maintaining sperm motility over time as well as identify one or more media to facilitate sperm sorting using flow cytometry. BIL, TES, and INR all contained a source of lipoprotein (BIL, 20% egg yolk; TES, 20% egg yolk; and INR, skim milk + 4% egg yolk). All extenders were prepared per manufacturer's instructions and stored at −20°C until use.

Semen Processing, Dilution, and Storage

Ejaculates exhibiting at least 60% total sperm motility were divided into 5 aliquots and slowly diluted with the respective extender treatments to a final concentration of 250×10^6 spermatozoa/mL. A control (CON) aliquot consisting of the undiluted raw ejaculate was also included in the analysis. To examine the effect of storage temperature, each aliquot was further divided into 3 equal aliquots (6 × 3 factorial arrangement) and incubated at 1 of 3 storage temperatures: 1) body (BODY; 35°C), 2) ambient (ROOM; 22°C), or 3) chilled (COLD; 4°C). BODY aliquots were maintained on a dry heating block set at 35°C. The ROOM aliquots were maintained in an insulated covered box. The COLD samples were placed in a water jacket (300 mL; 22°C) and held in a refrigerator (4°C) for more than 2.5 hours until they reached 4°C. For the duration of the study, the COLD samples were maintained at 4°C.

Analysis of Spermatozoa

To evaluate the effect of each diluent and storage temperature, an aliquant of semen was analyzed at 0, 1, 2, 4, 6, 12, and 24 hours. Briefly, 8 μL of semen from each treatment was placed on a prewarmed (35°C) glass slide, covered with a cover slip, and examined using a phase-contrast microscope. For each time period, %tMOT, %pMOT, and %INT were evaluated. Analysis for each storage temperature began (ie, time 0 hour) when samples reached their respective storage temperatures.

Table 1. *Seminal characteristics of Asian and African elephant bulls*

Seminal Characteristic	Asian Elephant (12 bulls; n = 75 ejaculates)[a]	African Elephant (4 bulls; n = 50 ejaculates)[a]
Total volume, mL	48.3 ± 58.3	30.1 ± 38.7
Total motility, %	25.8 ± 31.4	22.9 ± 30.3
Total progressive motility, %	15.7 ± 26.3	18.5 ± 28.6
Morphologically normal, %	56.5 ± 26.9	45.6 ± 25.6
Normal acrosome, %	17.3 ± 23.9	23.0 ± 31.0
Sperm concentration ($\times 10^6$)	1308.8 ± 794.9	2276.9 ± 706.7
pH	7.2 ± 1.1	7.2 ± 0.9
Osmolality, mOsm	256.2 ± 55.0	356.6 ± 124.4

[a] Values represent means ± SD. Ejaculates with urine contamination were not included in the analyses.

Table 2. *Seminal traits of Asian and African elephant ejaculates with ≥60% initial total motility*

Seminal Characteristic	Asian Elephant (4 bulls; n = 10 ejaculates)[a]	African Elephant (2 bulls; n = 5 ejaculates)[a]
Total volume, mL	123.9 ± 93.6	20.3 ± 12.0
Total motility, %	82.5 ± 8.9	87.0 ± 6.7
Total progressive motility, %	78.0 ± 7.6	87.0 ± 6.7
Morphologically normal, %	79.2 ± 13.1	67.3 ± 22.5
Normal acrosome, %	54.1 ± 12.7	78.2 ± 8.7
Sperm concentration ($\times 10^6$)	649.1 ± 633.4	2500.6 ± 934.2
pH	7.4 ± 0.7	7.5 ± 0.8
Osmolality, mOsm	253.5 ± 11.4	273.6 ± 17.7

[a] Values represent means ± SD.

Statistical Analysis

The experimental design was set in a 6 × 3 factorial arrangement, with 5 extender treatments (plus CON) and 3 storage temperature conditions. Treatment differences among extender treatments and storage temperatures were determined by analysis of variance using SAS (version 9.1; SAS institute Inc, Cary, North Carolina). A Shapiro-Wilk test was used to test for normality of the data. Generalized linear model analyses was used to determine the differences and the effects of bull (as a random effect), sample within bull, extender, temperature, and time on %tMOT, %pMOT, and %INT of elephant spermatozoa treated with different extenders and storage temperatures. The bull effect was included in the model to account for the variation among bulls. Significant differences between treatment means were determined using Tukey's honestly significant difference (HSD) test. Values were expressed as means ± SEM. The level of statistical significance was set at $P < .05$ for all hypotheses tested. Tukey's HSD test employed an experiment-wise error rate of protection for type I error.

Results

Seminal Traits

A total of 125 ejaculates were collected from 12 Asian (6–35 years; 7 institutions) and 4 African (8–31 years; 3 institutions) elephant bulls (Table 1). Ejaculates contaminated with urine (n = 110) were not included in the analyses. Mean (± SD) sperm %tMOT (Asian, 25.8% ± 31.4%; African, 22.9% ± 30.3%) and %pMOT (Asian, 15.7% ± 26.3%; African, 18.5% ± 28.6%) were low in raw ejaculates with more than 45% morphologically normal sperm (Table 1). Overall, only 10 of 75 ejaculates (13.3%) and 5 of 50 ejaculates (10%) from 4 Asian (14–35 years; 4 institutions) and 2 African (8–24 years; 2 institutions) elephant bulls, respectively, met the minimum quality criterion (≥60% %tMOT) to be used in the study (Table 2). Average %tMOT of ejaculates that were included in the study were 82.5% ± 8.9% and 87.0% ± 6.7% for Asian and African elephants, respectively.

Asian Elephant

Effect of Semen Extenders on Asian Elephant Sperm %tMOT, %pMOT, and %INT—At 0 hours, no differences in %tMOT, %pMOT, or %INT were observed among extenders (Table 3). At 4 hours, INR, BIL, and TES retained the highest %tMOT and %pMOT in Asian elephants. Dilution of semen in HEP or MOD resulted in a 50% decline in %tMOT from initial values. Although there was no difference in %pMOT among CON, HEP, or MOD, spermatozoa in MOD exhibited the lowest ($P < .05$) %pMOT compared with INR, BIL, and TES. The proportion of spermatozoa with normal acrosomes (%INT) was higher ($P < .05$) in INR and TES compared with HEP. At 6 hour, both egg yolk–based extenders, BIL and TES, maintained higher %tMOT ($P < .05$) compared with HEP and MOD, whereas INR and BIL exhibited higher %pMOT ($P < .05$) compared with HEP and MOD. A higher ($P < .05$) proportion of spermatozoa maintained INT in INR, BIL, and TES compared with HEP and MOD. When ejaculates were incubated for 12 or 24 hours, there were no differences ($P > .05$) among extenders in either %tMOT or %pMOT. However, BIL maintained higher %INT ($P < .05$) compared with CON, HEP, or MOD but was not different than INR or TES.

Diluted ejaculates maintained 45% and 27% of initial motility at 12 and 24 hours, respectively (Table 3). Overall, BIL, TES, and INR maintained higher %tMOT, %pMOT, and %INT values compared with other treatments through the duration of the study, maintaining 45%, 40%, and 38% of initial motility at 12 hours and 27%, 13%, and 14% at 24 hours, respectively. In contrast, sperm stored in HEP, MOD,

Table 3. *%tMOT, %pMOT, and %INT of Asian elephant spermatozoa stored as neat semen or in various extenders*[a]

	Time														
	0 h			4 h			6 h			12 h			24 h		
Extender	%tMOT	%pMOT	%INT	%tMOT	%pMOT	%INT	%tMOT	%pMOT	%INT	%tMOT	%pMOT	%INT	%tMOT	%pMOT	%INT
CON	67.1	61.3	34.8	42.3^{b,c}	33.8^{b,c,d}	23.6^{b,c}	32.1^{b,c,d,e}	23.8^{b,c,d}	18.8^{c,d}	15.8	8.1	10.2^{d}	0.4	0.0	3.0^{d}
HEP	59.8	54.6	33.1	29.3^{c}	20.8^{c,d}	20.0^{c}	23.8^{d,e}	13.8^{c,d}	17.2^{d}	11.0	3.8	8.6^{d}	2.2	0.0	3.1^{d}
MOD	62.1	55.5	35.2	25.5^{c}	10.2^{d}	26.6^{b,c}	19.8^{e}	5.7^{d}	23.3^{c,d}	8.4	0.3	16.4^{c,d}	2.0	0.0	9.8^{c,d}
INR[f]	70.4	65.3	38.2	57.3^{b}	54.1^{b}	29.1^{b}	46.3^{b,c,d}	42.0^{b}	26.7^{b,c}	26.8	21.3	20.3^{b,c}	10.1	6.5	14.2^{b,c}
BIL[f]	70.9	65.0	39.7	57.6^{b}	50.8^{b}	28.0^{b,c}	52.8^{b}	46.0^{b}	27.9^{b}	32.0	26.4	25.9^{b}	19.3	10.8	18.8^{b}
TES[f]	69.1	61.1	38.6	57.8^{b}	50.5^{b}	29.8^{b}	49.1^{b,c}	38.5^{b,c}	27.2^{b}	27.3	16.3	19.0^{b,c}	8.9	0.0	13.5^{b,c}
SEM	3.3	4.0	1.2	3.3	4.0	1.2	3.3	4.0	1.2	3.3	4.0	1.2	3.3	4.0	1.2

Abbreviations: BIL, Biladyl; CON, control (neat semen); HEP, TL-Hepes; INR, INRA96; MOD, Modena; TES, TEST refrigeration medium; %INT, percentage of acrosomal integrity; %pMOT, percentage of progressive forward motility; %tMOT, percentage of total sperm motility.

[a] Values represent means of 10 ejaculates.
[b,c,d,e] Within each column, values with different superscripts differ significantly ($P < .05$).
[f] Extender treatment is egg yolk or skim milk based.

and CON exhibited a greater loss in motility and maintained only 24%, 18%, and 14% of initial values at 12 hours and only 1%, 4%, and 3% of initial values remained by 24 hours, respectively.

Effect of Storage Temperature on Asian Elephant Sperm %tMOT, %pMOT, and %INT—At 0 hours, %tMOT and %pMOT were similar among all 3 storage temperatures (Table 4), but the proportion of spermatozoa with INT was higher ($P < .05$) at BODY and ROOM compared with aliquots stored at COLD. For all storage temperatures, a further decline was observed in all sperm parameters at 4 hours of storage, but no differences ($P > .05$) were observed among storage temperature treatments. At 6 hours, both %tMOT and %pMOT were higher ($P < .05$) at ROOM compared with BODY but similar to COLD. No differences ($P > .05$) were observed in the proportions of INT among storage temperatures. By 12 hours, %tMOT and %pMOT were abolished in aliquots stored at BODY, whereas aliquots stored at ROOM maintained 35.9% ± 2.4% %tMOT and 28.2% ± 2.8% %pMOT and COLD maintained 29.0% ± 2.4% %tMOT and 16.9% ± 2.8% %pMOT. At 12 and 24 hours, there were no differences ($P > .05$) in %tMOT and %pMOT between ROOM and COLD, and the proportion of spermatozoa with intact acrosomes was higher ($P < .05$) at ROOM and COLD compared with BODY.

Effect of Semen Extender and Storage Temperature on Asian Elephant Sperm %tMOT, %pMOT, and %INT—Among extenders maintained at BODY (Table 5), there were no differences in %tMOT, %pMOT, or %INT. At ROOM storage, BIL maintained higher ($P < .05$) %tMOT and %pMOT compared with MOD but was not different than CON, HEP, INR, or TES. Spermatozoa in BIL also maintained a higher ($P < .05$) proportion of %INT compared with HEP, but was not different ($P > .05$) than CON, MOD, INR, and TES. At COLD storage, semen extended in INR, BIL, or TES exhibited higher %tMOT and %pMOT compared with aliquots extended in HEP. Aliquots extended in INR also maintained higher ($P < .05$) %pMOT compared with HEP and MOD, but there were no differences compared with CON, BIL, or TES. The proportion of INT spermatozoa was higher ($P < .05$) in TES

Table 4. *%tMOT, %pMOT, and %INT of Asian elephant spermatozoa stored in 3 temperature conditions*[a]

	Time														
	0 h			4 h			6 h			12 h			24 h		
Storage Temperature	% tMOT	% pMOT	% INT	% tMOT	% pMOT	% INT	% tMOT	% pMOT	% INT	% tMOT	% pMOT	% INT	% tMOT	% pMOT	% INT
BODY (35°C)	72.5	65.7	41.1^{b}	41.1	32.0	26.2	26.5^{c}	17.6^{c}	23.0	0.0^{c}	0.0^{c}	10.4^{c}	0.0^{c}	0.0	4.2^{c}
ROOM (22°C)	70.9	66.8	38.3^{b}	54.6	47.3	28.7	49.4^{b}	41.7^{b}	26.5	35.9^{b}	28.2^{b}	22.8^{b}	7.5^{b,c}	1.2	13.8^{b}
COLD (4°C)	56.3	49.0	30.4^{c}	39.1	30.8	23.7	36.0^{b,c}	25.5^{b,c}	21.1	29.0^{b}	16.9^{b,c}	17.0^{b}	19.8^{b}	8.1	13.2^{b}
SEM	2.4	2.8	0.8	2.4	2.8	0.8	2.4	2.8	0.8	2.4	2.8	0.8	2.4	2.8	0.8

Abbreviations: %INT, percentage of acrosomal integrity; %pMOT, percentage of progressive forward motility; %tMOT, percentage of total sperm motility.

[a] Values represent means of 10 ejaculates.
[b,c] Within each column, values with different superscripts differ significantly ($P < .05$).

Table 5. *%tMOT, %pMOT, and %INT of Asian elephant spermatozoa stored as neat semen or in various extenders under different storage temperatures*[a]

	Storage Temperature								
	BODY (35°C)			ROOM (22°C)			COLD (4°C)		
Extender	%tMOT	%pMOT	%INT	%tMOT	%pMOT	%INT	%tMOT	%pMOT	%INT
CON	29.6[f]	24.0	22.9[e,f]	50.6[b,c,e]	45.8[b,c]	25.8[b,c,e]	36.0[b,c,e,f]	26.3[b,c,d]	14.5[d,f]
HEP	32.5[e,f]	28.1	21.1[e,f]	41.5[b,c,e]	32.2[b,c]	21.7[c,e]	20.7[c,f]	10.0[d]	14.1[d,f]
MOD	25.8	12.9	26.8	35.9[c]	24.4[c]	27.2[b,c]	29.1[b,c]	17.8[c,d]	21.6[c,d]
INR[g]	41.8	37.1	27.4	52.5[b,c]	49.0[b,c]	27.9[b,c]	52.0[b]	47.8[b]	29.1[b,c]
BIL[g]	44.5	38.2	27.8	60.8[b]	55.3[b]	33.6[b]	51.5[b]	43.1[b,c]	28.8[b,c]
TES[g]	42.9	35.3	24.8	57.0[b,c]	51.2[b,c]	29.3[b,c]	46.7[b]	33.7[b,c]	30.8[b]
SEM	2.2	2.6	0.8	2.2	2.6	0.8	2.2	2.6	0.8

Abbreviations: BIL, Biladyl; CON, control (neat semen); HEP, TL-Hepes; INR, INRA96; MOD, Modena; TES, TEST refrigeration medium; %INT, percentage of acrosomal integrity; %pMOT, percentage of progressive forward motility; %tMOT, percentage of total sperm motility.

[a] Values represent means of 10 ejaculates.
[b,c,d] Within each column, values with different superscripts differ significantly ($P < .05$).
[e,f] Within each row, values with different superscripts differ significantly ($P < .05$) between storage temperatures for each sperm parameter.
[g] Extender treatment is egg yolk or skim milk based.

compared with CON, HEP, and MOD, but there were no differences among INR, BIL, or TES. Likewise, %INT was similar among CON, HEP, and MOD.

Among storage temperatures, ROOM exhibited higher ($P < .05$) values in %tMOT, %pMOT, and %INT (Table 5). CON exhibited higher ($P < .05$) %tMOT at ROOM compared with BODY, whereas HEP maintained higher ($P < .05$) %tMOT at ROOM compared with COLD. The proportion of spermatozoa with INT in both CON and HEP was higher ($P < .05$) at ROOM compared with COLD but was not different than BODY. All other extender treatments (ie, BIL, INR, TES, and MOD) did not exhibit any differences in maintaining sperm survival regardless of storage temperature.

African Elephant

Effect of Semen Extenders on African Elephant Sperm %tMOT, %pMOT, and %INT—Similar to Asian elephant bulls, spermatozoa from their African counterparts also exhibited a decline over time (0 hours vs 24 hours) in %tMOT, %pMOT, and %INT (Figure). However, no differences in sperm parameters were observed among extenders within each time interval.

Effect of Storage Temperature on African Elephant Sperm %tMOT, %pMOT, and %INT—At 0 hours, all sperm parameters were similar among storage conditions (Table 6). Between 4 and 24 hours, the %tMOT, %pMOT, and %INT were higher ($P < .05$) at ROOM compared with BODY, and these parameters remained similar at COLD. At 12 hours and 24 hours, all sperm parameters evaluated were higher ($P < .05$) at ROOM and COLD compared with BODY.

Effect of Semen Extender and Storage Temperature on African Elephant Sperm %tMOT, %pMOT, and %INT—At BODY, there were no differences in %tMOT and %INT among extenders (Table 7). HEP and TES maintained a higher ($P < .05$) %pMOT compared with MOD at BODY storage, but values remained similar to those with CON, INR, and BIL. At both ROOM and COLD, there were no differences ($P > .05$) among extender treatments in maintaining %tMOT, %pMOT, and %INT for each storage temperature.

Among storage temperatures, there was a difference among extenders in their ability to maintain %tMOT, %pMOT, and %INT (Table 7). HEP and TES were effective at maintaining %tMOT, %pMOT, and %INT across all storage temperatures. CON, MOD, INR, and BIL exhibited an interaction with storage temperature and exhibited lower values in %tMOT, %pMOT, or %INT when stored at BODY. Specifically, CON, MOD, INR, and BIL exhibited lower ($P < .05$) %tMOT values at BODY compared with COLD but remained similar to that at ROOM. CON also exhibited lower ($P < .05$) values in %pMOT and %INT when sperm were stored at BODY compared with ROOM ($P < .05$) but was not different than COLD. In addition, %pMOT values were lower ($P < .05$) for MOD and INR at BODY compared with COLD, and %INT values were lower ($P < .05$) for BIL and INR at BODY compared with COLD.

Discussion

This study represents the first comprehensive assessment of liquid storage on both Asian and African elephant semen parameters and has resulted in several major findings, including 1) inherent differences in sperm sensitivity to components of various semen extenders

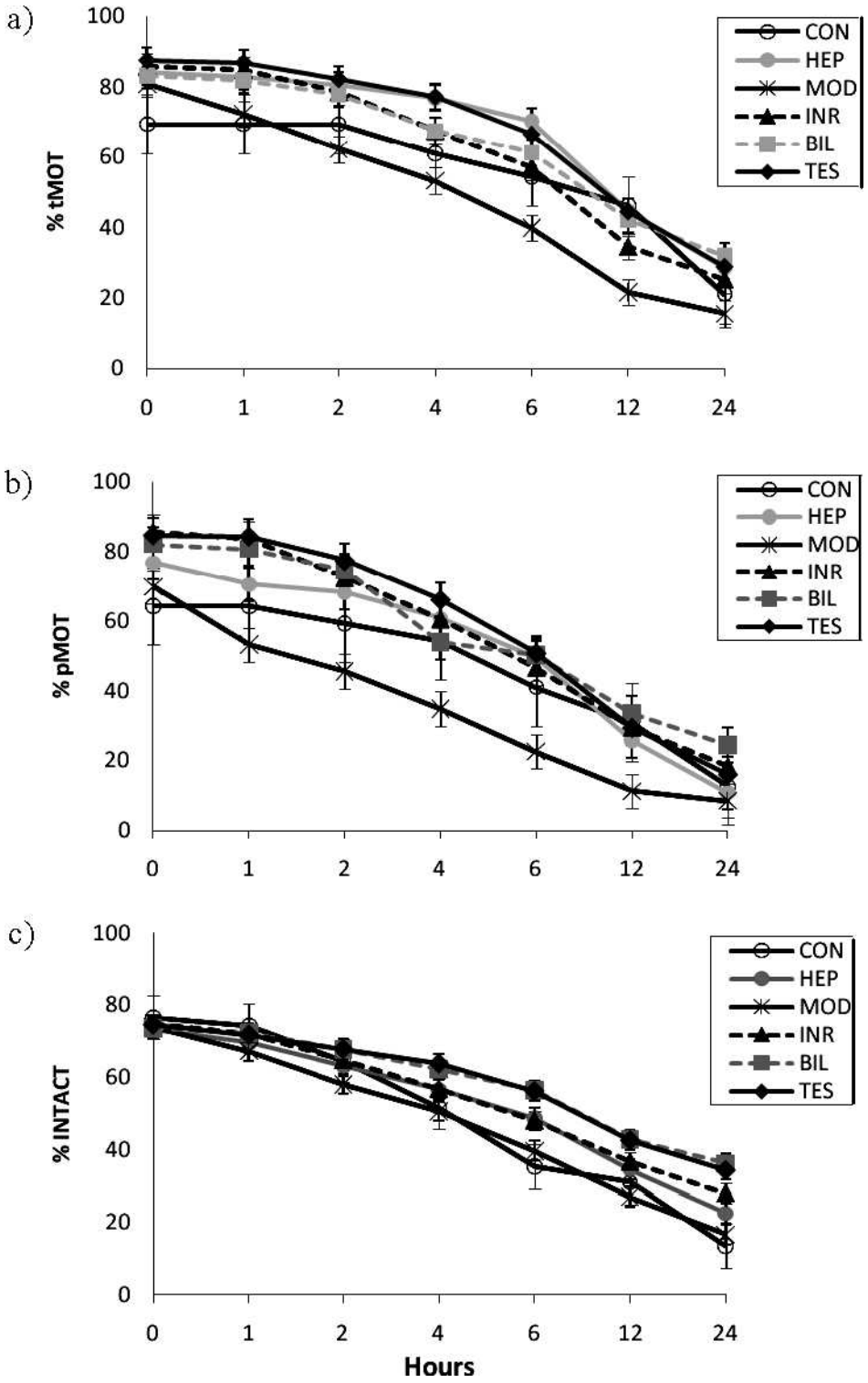

Figure. Percentage of **(a)** total sperm motility (%tMOT), **(b)** progressive forward motility (%pMOT), and **(c)** intact acrosomal status (%INTACT) of African elephant spermatozoa stored as neat semen (control [CON]) or in various extenders. HEP indicates TL-Hepes; MOD, Modena; INR, INRA96; BIL, Biladyl; TES, TEST. Values represent means ± SEM.

Table 6. *%tMOT, %pMOT, and %INT of African elephant spermatozoa stored in 3 temperature conditions*[a]

	Time														
	0 h			4 h			6 h			12 h			24 h		
Storage Temperature	% tMOT	% pMOT	% INT	% tMOT	% pMOT	% INT	% tMOT	% pMOT	% INT	% tMOT	% pMOT	% INT	% tMOT	% pMOT	% INT
BODY (35°C)	81.0	77.7	74.0	51.4[c]	39.7[c]	47.0[c]	41.2[c]	26.9[c]	33.9[c]	5.5[c]	3.9[c]	14.8[c]	0.7[c]	0.6[c]	5.6[c]
ROOM (22°C)	84.4	84.7	76.0	77.5[b]	72.1[b]	63.2[b]	70.5[b]	61.7[b]	55.2[b]	51.1[b]	36.6[b]	44.8[b]	26.9[b]	17.7[b]	32.2[b]
COLD (4°C)	80.0	69.4	73.4	72.7[b,c]	53.4[b,c]	60.6[b,c]	62.9[b,c]	41.6[b,c]	52.9[b]	60.0[b]	39.7[b]	47.7[b]	47.9[b]	27.1[b]	38.0[b]
SEM	3.4	4.5	2.5	3.4	4.5	2.5	3.4	4.5	2.5	3.4	4.5	2.5	3.4	4.5	2.5

Abbreviations: %INT, percentage of acrosomal integrity; %pMOT, percentage of progressive forward motility; %tMOT, percentage of total sperm motility.

[a] Values represent means of 5 ejaculates.
[b,c] Within each column, values with different superscripts differ significantly ($P < .05$).

and storage temperatures, 2) spermatozoa from African elephants maintained both %tMOT and %pMOT longer than their Asian counterparts, 3) storage at temperatures lower than BODY improved sperm survival, 4) media containing a source of lipoprotein (ie, egg yolk [BIL or TES] or skim milk supplemented with 4% egg yolk [INR]) are better able to maintain Asian elephant sperm %tMOT and %pMOT than other extenders, and 5) African elephant sperm are less discriminating and tolerate all storage temperature (35°C, 22°C, or 4°C) conditions and survive best in HEP (tissue culture medium) or TES (containing 20% egg yolk). Collectively, the data demonstrated that both extender composition and storage temperature influence longevity of elephant spermatozoa.

A major challenge to the study of male reproduction in elephants is the unpredictability of ejaculate quality. In the present study, less than 13% (Asian) and 10% (African) of the ejaculates exhibited at least 60% sperm motility and rendered them suitable as test ejaculates. %tMOT, %pMOT, and %INT were highly variable among ejaculates from both species. Intraindividual and interindividual variations in semen quality have previously been reported in elephants (Howard et al, 1984; Mar et al, 1993; Thongtip et al, 2004; Saragusty et al, 2005; Portas et al, 2007; Thongtip et al, 2008; Saragusty et al, 2009b). Factors such as method of semen collection, frequency of ejaculation, length of time since last emission, hormonal and diurnal rhythms, temperature and light conditions, season, and nutrition may influence ejaculate quality (Mann and Lutwak-Mann, 1981; Goeritz et al, 2003; Thongtip et al, 2008), perhaps as a result of variations in seminal plasma composition (ie, volume, pH, osmolality, proteins, macroelements, and microelements) that might have repercussions on various aspects of sperm structure and function (Mann and Lutwak-Mann, 1981; Topfer-Petersen et al, 2005; Yoshida et al, 2008). Investigations are underway in our

Table 7. *%tMOT, %pMOT, and %INT of African elephant spermatozoa stored as neat semen or in various extenders under different storage temperatures*[a]

	Storage Temperature								
	BODY (35°C)			ROOM (22°C)			COLD (4°C)		
Extender	%tMOT	%pMOT	%INT	%tMOT	%pMOT	%INT	%tMOT	%pMOT	%INT
CON	35.6[e]	34.5[b,c,e]	37.3[e]	69.9[d]	65.2[d]	60.3[d]	62.0[d]	40.2[d,e]	51.2[d,e]
HEP	57.9	52.9[b]	45.1	75.1	63.6	58.1	67.4	38.8	54.7
MOD	35.4[e]	14.8[c,e]	40.7	50.8[d,e]	46.3[d]	49.5	61.8[d]	44.2[d]	52.6
INR[f]	45.2[e]	38.9[b,c,e]	40.1[e]	66.8[d,e]	63.9[d,e]	58.3[d]	73.8[d]	67.3[d]	64.9[d]
BIL[f]	48.1[e]	41.9[b,c]	48.3[e]	68.8[d,e]	64.6	63.1[d,e]	74.1[d]	64.6	64.8[d]
TES[f]	56.5	52.3[b]	50.1	76.0	70.1	65.6	70.5	53.1	60.5
SEM	2.4	3.2	1.8	2.4	3.2	1.8	2.4	3.2	1.8

Abbreviations: BIL, Biladyl; CON, control (neat semen); HEP, TL-Hepes; INR, INRA96; MOD, Modena; TES, TEST refrigeration medium; %INT, percentage of acrosomal integrity; %pMOT, percentage of progressive forward motility; %tMOT, percentage of total sperm motility.

[a] Values represent means of 5 ejaculates.
[b,c] Within each column, values with different superscripts differ significantly ($P < .05$).
[d,e] Within each row, values with different superscripts differ significantly ($P < .05$) between storage temperatures for each sperm parameter.
[f] Extender treatment is egg yolk or skim milk based.

laboratory to correlate seminal plasma composition (eg, chemistry and proteins) with ejaculate quality in both elephant species.

Asian elephant spermatozoa exhibited a faster decline and lower %tMOT, %pMOT, and %INT over a shorter duration compared with African elephant spermatozoa. After 24 hours of semen storage, maximum sperm motility was 19.8% ± 2.4% compared with 47.9% ± 3.4%, and %INT was 13.8% ± 0.8% compared with 38.0% ± 2.5% for Asian (Table 4) and African (Table 6) elephants, respectively. The faster loss in sperm survival exhibited by Asian elephant spermatozoa demonstrated an overall greater sensitivity to in vitro storage and chilling compared with their African counterparts and is a major concern because of the unacceptable semen quality.

The differences in sperm membrane structure between the 2 elephant species have been suggested to be responsible for the disparity in sperm survival rates during in vitro semen storage (Swain and Miller, 2000). The composition and combination of various membrane components, including cholesterol, phospholipids, saturated and unsaturated fatty acids, and proteins, characterize the intrinsic properties and fluidity of the sperm membrane and determine its adaptability to temperature phase transitions during cooling (Darin-Bennett and White, 1977; Quinn et al, 1980; Watson and Morris, 1987; Hammerstedt et al, 1990; Parks, 1997; Cross, 1998; Giraud et al, 2000; de Albuquerque Lagares et al, 2008). Swain and Miller (2000) determined that African elephant spermatozoa contain higher levels of long-chain polyunsaturated fatty acid (eg, docosahexaenoic acid) in their membranes compared with Asian elephant spermatozoa. Perhaps the differences in polyunsaturated fatty acids, in addition to other membrane properties and factors such as cholesterol and phospholipid ratios, may exert a synergistic influence on the susceptibility of spermatozoa during cooling and warrants further investigation.

In the present study, media containing a source of lipoprotein (BIL, TES, and INR) protected spermatozoa during cooling. For Asian elephants, the inclusion of egg yolk or milk was a necessary component in extenders to support sperm survival as evidenced by the precipitous decline in the parameters measured as a function of non–egg yolk–containing extenders. In contrast, African elephant spermatozoa were less discriminating, surviving just as well in media that did not contain egg yolk (eg, HEP, MOD). These results agree with earlier reports in which egg yolk (Graham et al, 2004; Saragusty et al, 2005) and skim milk–based media (Hermes et al, 2009) better preserved Asian elephant spermatozoa during cooling and non–egg yolk media was effective at short-term liquid storage of African elephant spermatozoa (Jones, 1973).

The protective effect of egg yolk in semen extenders has been known for decades (Phillips and Lardy, 1940), and its ability to enhance sperm survival at a broad range of temperatures, including ambient (Dunn et al, 1950; Shannon and Curson, 1983) and lower temperatures (Phillips and Lardy, 1940; Watson, 1981; de Leeuw et al, 1993; Amirat et al, 2004), has allowed it to be successfully used in a variety of species (Parks and Graham, 1992). The value of egg yolk is particularly evident during cooling and freezing, when its presence reduces the severity and extent of cold shock on spermatozoa (Medeiros et al, 2002; Saragusty et al, 2005), perhaps by a mechanism whereby phospholipid low-density lipoproteins coat and enhance the stability and integrity of the sperm plasma membrane, which in turn confers protection on spermatozoa (Watson, 1975; Quinn et al, 1980; Blanchard et al, 1998; De Pauw et al, 2003). The presence of lipoprotein in extension media may also prevent an efflux of membrane cholesterol that destabilizes sperm and is deleterious to their survival (Bergeron et al, 2004; Bergeron and Manjunath, 2006).

One of the major objectives of this study was to identify an extender that will optimize short-term storage of elephant spermatozoa while simultaneously being compatible with the needs of sperm sex-sorting technology. Egg yolk–based extenders have been used in the past to preserve Asian and African elephant spermatozoa because the inclusion of egg in extenders is known to enhance sperm survival during in vitro semen storage. However, egg yolk must be removed before sperm sex sorting because it interferes with the uniform staining of DNA that is critical to separate X and Y chromosome–bearing populations of sperm (Johnson and Welch, 1999). Further processing and handling to remove remnants of egg yolk before sperm sex sorting can cause additional sperm damage and loss in sperm viability to an already vulnerable sperm population. For these reasons, non–egg yolk– or reduced egg yolk–based extenders (ie, MOD, HEP, and INR), which do not require additional processing for sperm sex sorting, were included in this study to determine their efficacy at maintaining sperm survival for both Asian and African elephants. For Asian elephants, INR, a skim milk medium supplemented with only 4% egg yolk sustained sperm as well as media containing 20% egg yolk and thus can be safely used for extending ejaculates and shipment to centralized sperm-sorting facilities. A similar conclusion was recently reached by Hermes et al (2008) who reported that a skim milk–based medium was ideal for storage and sex sorting of Asian elephant sperm. For African elephants, this was the first study to systematically investigate an

optimal semen extender for sperm sex sorting. We found that HEP maintained sperm survival at all storage temperatures, whereas MOD was the most effective extender for cold storage. Because neither HEP nor MOD contain egg yolk, it appears that either could safely be used for extending African elephant ejaculates and shipment to sorting facilities. However, because these results were based only on ejaculates from 2 donors, additional studies with more bulls are warranted to confirm these findings.

In both species, semen storage at body temperature was detrimental regardless of extender treatment, and spermatozoa demonstrated a precipitous decline in all parameters after 4 hours of storage. This was particularly evident for the storage at 35°C of raw semen, which exhibited lower semen parameter values compared with those at 22°C and 4°C. As a result, storage of both Asian and African elephant semen at body temperature (35°C) should be avoided, and storage at 22°C and 4°C is recommended. Similar results also have been reported in cattle for which semen stored at a temperature above refrigeration (4°C) exhibited reduced values in both sperm survival and fertility (Vishwanath and Shannon, 1997), with semen storage at body temperature exhibiting the lowest values (Bartlett and Van Demark, 1962). The decline in sperm survival at 35°C can be attributed to the increased energy consumption and rate of metabolism that spermatozoa undergo at warmer temperatures. Exposing spermatozoa to cooler temperatures reduces the rate of consumption and metabolism, thus allowing sperm to be preserved for a longer duration (Blackshaw et al, 1957; Bartlett and Van Demark, 1962; Clarke et al, 1982; Gadea, 2003). Interestingly, for both species, storage at 22°C was no different than storage at 4°C. This is of economical and practical importance, for it suggests that elephant semen can be collected and preserved under field conditions where electricity, refrigeration, or liquid nitrogen may not be immediately available for cooling or freezing. However, it is important to mention that although we did not find significant differences in sperm motility and acrosome status between 22°C and 4°C over the duration of our study, perhaps a significant difference may exist between the 2 storage temperatures following more than 24 hours of semen storage.

In summary, our study of the sensitivity of Asian and African elephant spermatozoa to various semen extenders and storage temperatures suggests differences between the 2 species in the ability of spermatozoa to survive during in vitro storage. African elephant spermatozoa were more robust in maintaining motility compared with Asian elephant spermatozoa. Under all conditions evaluated, it was best to store semen at temperatures lower than body temperature. Furthermore, Asian elephant spermatozoa were preserved better in extenders containing egg yolk (BIL and TES) or skim milk (INR), whereas spermatozoa from their African counterparts did not have such a requirement and survived just as well in buffers containing no egg yolk or skim milk (MOD and HEP). These findings advance our understanding of elephant sperm sensitivity to extenders and storage conditions and could improve our overall ability to implement sound genetic management practices to ensure the long-term survival of elephants in captivity and in the wild.

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