SPERM SORTING AND PRESERVATION TECHNOLOGIES FOR SEX RATIO MODIFICATION IN THE ELEPHANT AND RHINOCEROS: AN UPDATE

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Abstract

Management of sustainable *ex situ* populations of elephants and rhinoceroses would be enhanced by the ability to modify the sex ratio of offspring. A bias of the sex ratio towards females, through the insemination of cows with semen samples enriched for X chromosome-bearing spermatozoa, would improve the population's reproductive potential, reduce the number of males, and thereby facilitate the housing of socially cohesive groups. Sperm sexing technology could also address undesirable sex ratio skews and when combined with sperm preservation methodologies and effective genome storage banks, would aid in the cooperative management and long-term conservation of these threatened species. Sperm sorting and preservation research, which has been endorsed by the AZA TAG/SSP Programs of both taxa, has been conducted by our group and collaborators over the past six years. The goal is to adapt flow cytometry technology to sort elephant and rhinoceros spermatozoa and produce fresh and frozen-thawed X-enriched samples of adequate quality for artificial insemination (AI). Standard methods of semen collection in the elephant (manual stimulation of standing males) and rhinoceros (electroejaculation [EEJ] of anaesthetized males) both produce samples exhibiting great variation in quality. For the Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant, results demonstrated that initial ejaculate quality must meet specific *in vitro* criteria to allow the subsequent production of high quality sorted spermatozoa. Initial ejaculate criteria necessary for overnight shipment to the sorting facility were determined ("viable samples" >70% progressive motility and plasma membrane integrity, < 15% detached heads, < 0.013 mg creatinine/ml). When viable samples were utilized, *in vitro* quality of sex-sorted, chilled Asian and African elephant spermatozoa was well maintained over a 24 h period, and samples were considered adequate for use in AI trials if inseminated within 24 h after sorting. Despite these promising results, the proportion of ejaculates designated as viable were low for both elephant species. Only 16% (19/118) of manual stimulation collection attempts yielded viable samples from 10 males in the North American and Australasian populations. For the three rhinoceros species examined (white: *Ceratotherium simum,* black: *Diceros bicornis*, Indian: *Rhinoceros unicornis*), the most progress has been made with the former species. White rhinoceros spermatozoa derived from viable electroejaculates displayed *in vitro* characteristics that were considered suitable for

use in AI, after sorting and chilled storage for 36 h, and after cryopreservation and thawing using directional freezing technology. Across all three rhinoceros species, the proportion of samples of adequate quality for sorting and preservation after EEJ of 11 males in the North American population was moderate (40%, 8/20), but final numbers of spermatozoa from viable samples available for sorting was highly variable (2059 ± 1932 x 10^6 spermatozoa/male). In summary, methods for sex-sorting and chilled storage of spermatozoa (prior to and after sorting) have been established for both elephant species. Sex-sorting, chilled storage and cryopreservation methods have also been developed for the white rhinoceros. Inconsistent ejaculate quality is the greatest challenge to producing the substantial numbers of sex-sorted spermatozoa necessary for an insemination dose in all of the aforementioned species.

Introduction

The sustainability of *ex situ* elephant and rhinoceros populations relies on maximizing reproduction rates during each individual's functional window of fertility and ensuring maintenance of existing genetic diversity. Assisted reproductive technologies such as artificial insemination (AI) and semen preservation have been under development to assist with the reproductive and genetic management of these species[eg3,5,7,9,12,14,21,24,26,27] More than 23 elephants (*Elephas maximus* and *Loxodonta africana*) have been born during 1999-2008 as a result of AI with chilled semen, but a sex ratio skew has been reported, with 83% of calves being male[22]. Though this male bias may lessen over time as more AI calves are born, it is detrimental to the long-term sustainability of the small *ex situ* elephant population. In the rhinoceros, naturally occurring skews towards males exist for some species (black rhinoceros, *Diceros bicornis*[20]). A bias towards females could address such skews and would be beneficial to rhinoceros and elephant captive breeding program by increasing the population growth rate and reducing the requirement for the housing of surplus males.

Predetermination of the sex of offspring can be accomplished by inseminating females with sex-sorted spermatozoa[10]. The utility of this technology is further enhanced by the preservation of sexed spermatozoa, for integration into genome resource banks and systematic use in AI or IVF and embryo transfer (reviewed by[16]). Consequently, sperm sorting and preservation research has been endorsed by the AZA TAG/SSP programs of elephants and rhinoceroses, to determine the feasibility of these technologies for use in species' management and conservation.

The long-term goal of this research is to adapt flow cytometry technology to sort elephant and rhinoceros spermatozoa and produce fresh and frozen-thawed X-enriched samples of adequate quality for AI. The specific objectives of elephant and rhinoceros sperm sorting and preservation research outlined in this presentation were to: (i) establish semen collection and pre-sorting storage methods, and initial ejaculate quality necessary for the production of high quality sex-sorted spermatozoa; (ii) determine the *in vitro* quality of sex-sorted spermatozoa after short-term preservation (chilled storage) and; (iii) examine the *in vitro* quality of sex-sorted spermatozoa after long-term preservation (cryopreservation; results are presented for the white rhinoceros, *Ceratotherium simum simum,* only).

Methods

Semen collection procedures described within were reviewed and approved by collaborating institutions' Institutional Animal Care and Use Committees.

Reagents and media

All chemicals were of analytical grade and cell culture tested where possible by the manufacturer. Unless stated otherwise, all media components were purchased from Sigma Aldrich (Sigma, St Louis, MO, USA) and were prepared with tissue culture-grade water (Sigma or Millipore, Billerica, MA, USA). Diluents containing egg yolk (free range eggs) were prepared by ultracentrifugation for 1.5 h at 10,000 g. The supernatant was filtered (0.22 µm; Millipore) and frozen at -80°C for a maximum of 18 months.

Animals and Semen Collection

Elephant

Semen collection attempts (n = 118) were made during 2006-2011 from 10 adult bulls (8 *Elephas maximus* [Ema], 2 *Loxodonta Africana* [Laf]) in the North American and Australasian region. Semen was collected by rectal massage of the pelvic urethra, ampulla glands and vas defera as described previously[17,23]. When available, transrectal ultrasound was used to examine the accessory glands prior to semen collection, in particular the ampullae, to ensure an adequate semen reservoir was present and avoid overstimulation and urination[8]. "Clean out" collections were also performed several days prior to experiments to assist in minimizing the proportion of aged spermatozoa (cells with heads detached from the midpiece/tail) in the ejaculate. The penis was washed with warm (~30°C) tap water followed by a HEPES-TALP medium to remove debris and residue urine, then dried with sterile cotton gauze. Semen was collected into sterile WHIRL-PAK bags (NASCO, Fort Atkinson, WI) containing 3 ml of an in-house prepared diluent (Table 1). Raw semen volume was calculated for each collection bag and an appropriate volume of elephant diluent was added to achieve a final dilution rate of 1:2 (semen: collection medium, v/v).

Trials were conducted to establish minimum initial *in vitro* sperm characteristics and appropriate processing methods (chilled storage, staining and sheath media) that enabled optimum resolution of X- and Y-sperm populations and *in vitro* sperm quality. Different semen transport diluents (supplemented with various egg yolk concentrations), staining and sheath media were examined. Based on *in vitro* sperm characteristics post-transport and post-staining, and resolution of X and Y sperm populations during sorting, an optimum method was developed for each species (as described in the results section).

Rhinoceros

Semen collection attempts (n = 20) were made during 2006-2011 from 11 adult bulls (5 *Diceros bicornis michaeli* [Dbi], 3 *Ceratotherium simum simum* [Csi], 3 *Rhinoceros unicornis* [Run]) in the North American region. Semen was collected by electroejaculation (EEJ) of anesthetized males[19]. The penis was rinsed with warm (~30°C) tap water followed by a HEPES-TALP

medium to remove debris and residue urine, then dried with sterile cotton gauze. A lubricated rectal probe, designed specifically for the rhinoceros, was used to conduct an electrical current for stimulation of accessory sex glands and semen emission. A collection series, consisting of 3 to 5 sets of 10 to 30 stimulations (voltage range: 2-12 V), typically resulted in semen emission. Manual rectal massage was administered during the 5 min rest periods between series. Semen was collected into sterile whirlpak bags containing 2 ml of an in-house prepared diluent (Table 1). Raw semen volume was calculated for each collection bag and an appropriate volume of rhino diluent was added to achieve a final dilution rate of 1:2 [Run] or 1:3 to 1:10 [Dbi, Csi] (semen: collection medium, v/v).

Semen transport

Semen was transported to the sorting facility at SeaWorld and Busch Gardens Reproductive Research Center in San Diego (CA, USA) within 12 to 23 h of collection using a modified Equitainer® (Hamilton Research, South Hamilton, MA, USA; final temperature after cooling was 9–12°C) placed in a box lined with stryofoam packing material for additional insulation against temperature fluctuations. After transport, semen was held at 12°C until processing for flow cytometric sorting.

Flow cytometric analysis and sorting

Semen was prepared for sorting by dilution with staining medium (Table 1) to achieve a concentration of 50 to 200 x 10^6 spermatozoa/ml. Spermatozoa were incubated with 40.1-97.9 µM H33342 for 45 min at 33.5°C. Immediately before sorting, stained samples were diluted (1:0.5, v/v) with staining medium containing 0.003% food dye (FD&C #40; Warner Jenkinson, St Louis, MO, USA) and filtered (35 µm). A high-speed cell sorter (MoFlo®SX, Dako, Fort Collins, CO, USA) modified for sperm sorting (reviewed by[25]) was used to analyze and separate spermatozoa. Reanalysis of sorted samples (sort reanalysis[11]) was used to evaluate accuracy of sorting.

Semen preservation

Chilled storage trials were conducted using sorted or control (non-sorted) spermatozoa. After sorting, spermatozoa were centrifuged (750 g, 12-15 min), resuspended in catch medium (Table 1) and slowly cooled (~-0.2°C/min) to 5°C. A cryopreservation trial was performed using sorted and non-sorted Csi spermatozoa. Spermatozoa were frozen using a previously described rhino cryodiluent (EQ, Table 1, with 2.5% Equex STM[14]) and directional freezing[1].

Semen and sperm evaluations

Raw and diluted semen was assessed for pH, osmolality and creatinine concentration (Jaffe reaction). Raw semen samples were collected from the inside wall of the collection bag prior to mixing with collection diluents when possible. A correction factor was applied to diluted samples for the calculation of creatinine concentrations and the creatinine threshold (collection diluents contained 0.008-0.013 mg creatinine/ml). Sperm motility, kinetic rating and agglutination rating of diluted samples were assessed subjectively within 20 min of semen

collection. Sperm concentration was determined from diluted samples after transport, as were motility parameters (Computer Assisted Sperm Analysis, Hamilton–Thorne, HTM-IVOS]), acrosome integrity and viability (dual stain method using propidium iodide and FITC-PNA[18]; validated for pachyderms *O'Brien, unpublished*) and morphology (glutaraldehyde fixation [raw ejaculates] and eosin-nigrosin [post-sorting]). *In vitro* assessments were also made after sorting/chilled storage (elephant: 0 h, 6 h, 24 h; rhinoceros: 0 h, 12h, 18 h, 36 h), and after sorting and cryopreservation (rhinoceros, Csi: 0 h and 3 h post-thaw).

Statistical analysis

Sperm quality data were analyzed using ANOVA (SigmaStat®, Version 2.0; SSPS, Inc., San Rafael, CA, USA). Prior to ANOVA, data were normalized by log-transformation when necessary. All pairwise multiple-comparison procedures between means were conducted using the Tukey test. $P < 0.05$ was considered to be significant. Data are presented as untransformed means ± SD.

Results

Establish semen collection and pre-sorting storage methods, and initial ejaculate quality necessary for the production of high quality sex-sorted spermatozoa

Elephant

The optimum medium used for the collection and transport of elephant semen to the sorting facility is shown in Table 1. Initial ejaculate quality criteria necessary for overnight shipment to the sorting facility and subsequent production of high quality sorted samples ("viable samples") were: >70% progressive motility and plasma membrane integrity, <15% detached heads and <0.013 mg creatinine/ml (of diluted samples). Samples exceeding threshold values of percent detached heads and creatinine concentration displayed reduced *in vitro* quality post-sorting (< 40% progressive motility, < 75 µm/s average path velocity). The percentage of collection attempts that produced ejaculates designated as viable was 15.5% (11/71) for Ema and 17.0% (8/47) for Laf. For each species, all viable samples were from one male. These males produced 11/34 (24.2%) and 8/33 (32.4%) viable samples per collection attempts, respectively. The final number of spermatozoa from viable samples available for sorting was $1768 \pm 1161 \times 10^6$ spermatozoa for Ema, and $49539 \pm 43944 \times 10^6$ spermatozoa for Laf. Good resolution of X- and Y-sperm populations was achieved for Ema and Laf males. Sort reanalysis results revealed high levels of purity for X- and Y-enriched samples obtained using moderate sorting rates for Ema (X-sperm: 89.3 ± 1.5%, 2000 ± 173 sperm/s; Y-sperm: 87.7 ± 0.6%, 1667 ± 58 sperm/s, n = 4) and Laf (X-sperm: 92.7 ± 3.4%, 2067 ± 249 sperm/s; Y-sperm: 88.5 ± 1.5, 1800 ± 163 sperm/s, n = 4).

Rhinoceros

The optimum medium used for the collection and transport of rhinoceros semen to the sorting facility is shown in Table 1. Similar to the elephant, rhinoceros electroejaculates needed to meet the same specific *in vitro* criteria prior to transport to enable the subsequent production of high quality sex-sorted spermatozoa. Samples exceeding threshold values of percent detached heads

and creatinine concentration displayed reduced *in vitro* quality post-sorting (< 40% progressive motility, < 50 µm/s average path velocity). The proportion of samples of adequate quality for sorting and preservation after EEJ was low for Dbi (2/9, 22.2%), moderate for Csi (4/8, 50.0%) and high for Run (2/3, 66.7%) males. Final numbers of spermatozoa from viable samples available for sorting varied across all species (Csi: 517 ± 428 x 10^6 spermatozoa; Dbi: 4296 ± 807 x 10^6 spermatozoa; Run: 3661 ± 1319 x 10^6 spermatozoa). Good resolution of X- and Y-sperm populations was achieved for Csi and Dbi males whereas resolution of Run samples was achieved only after the removal of egg yolk prior to sorting. Removal of egg yolk caused a drastic reduction in the *in vitro* quality of Run samples (results not shown). X- and Y-enriched samples obtained using moderate sorting rates displayed high levels of purity for Csi (X-sperm: 89.0 ± 11.3%, 2160 ± 509 sperm/s; Y-sperm: 88.5 ± 7.8%, 1755 ± 827 sperm/s, n = 4) and Dbi (X-sperm: 93.3 ± 6.4%, 2040 ± 316 sperm/s; Y-sperm: 83.7 ± 1.5%, 1830 ± 599 sperm/s, n = 2) males.

Table 1. Optimum diluents for the collection, transport and sorting of elephant and rhinoceros spermatozoa. All diluents were prepared in-house and were supplemented with antibiotics (315 ± 5 mOsm/kg, pH 7.1 ± 0.1).

Species	Diluent	Diluent composition	Diluent use[1]	Reference
Elephant	TEST-yolk buffer (TYB)	TES (176 mM) Tris (80 mM) Fructose (9 mM) Na-pyruvate (0.4 mM) BSA (0.15% v/v) Egg yolk (10% v/v) Gentamicin sulfate (50 µg/ml) Tylosin (8 µg/ml) Lincomycin (0.1 mg/ml) Spectinomycin (0.2 mg/ml) 315 ± 5 mOsm/kg, pH 7.3 ± 0.1	Collection medium Staining medium Sheath fluid Catch medium	Modified from[4]
Rhinoceros	EQ	Lactose (monohydrate, 171 mM) Glucose (89 mM) Tri Na-citrate (dihydrate, 3.7mM) EDTA (disodium, 2.7 mM) Na-bicarbonate (4.2 mM) Equex STM (0.125%) Egg yolk (10% v/v) Penicillin-G (0.0154 mg/ml) Streptomycin (0.0333 mg/ml) 315 ± 5 mOsm/kg, pH 7.1 ± 0.1	Collection medium Staining medium Sheath fluid Catch medium	Modified from[13,14]

[1]Staining medium and sheath fluid were prepared without egg yolk for staining and sorting processes, and tylosin, lincomycin and spectinomycin were replaced with Penicillin-G and Streptomycin sulphate. Staining medium contained 0.15% v/v BSA. Catch medium contained 20% v/v egg yolk.

In vitro quality of sex-sorted spermatozoa after short-term preservation (chilled storage)

Elephant

In vitro quality of sex-sorted, chilled Ema and Laf spermatozoa derived from ejaculates meeting aforementioned *in vitro* quality criteria was well maintained over a 24 h period (Table 2 and 3; results presented for 0 h and 24 h time-points only).

Table 2. Characteristics of sorted and non-sorted Asian elephant sperm (n = 3 ejaculates from 1 male) undergoing liquid storage at 5°C (n=9 replicates per treatment group).

Parameter	*Sorted Spermatozoa (15 x 10^6 spermatozoa/ml)*	*Non-sorted Spermatozoa (15 x 10^6 spermatozoa/ml)*
0 h Post-Sorting &/or Storage at 5 ºC		
Total motility (%)	84.5 ± 4.6[a]	62.3 ± 8.2[b]
Progressive motility (%)	44.7 ± 8.0[a]	13.1 ± 4.0[b]
Velocity (average pathway velocity, µm/s)	112.2 ± 3.8	104.5 ± 9.4
Agglutination rating (0-5)[A]	0.8 ± 0.4	0.8 ± 0.4
Intact plasma membrane & acrosome (%)[B]	79.3 ± 5.5[a]	68.3 ± 5.3[b]
Normal morphology (%)[C]	84.3 ± 3.8	78.2 ± 7.6
24 h Post-sorting &/or Storage at 5 ºC		
Total motility (%)	55.6 ± 16.2[a]	42.2 ± 12.8[b]
Progressive motility (%)	13.9 ± 8.8	10.9 ± 3.2
Velocity (average pathway velocity, µm/s)	78.3 ± 5.9	71.8 ± 16.2
Agglutination rating (0-5)[A]	0.7 ± 0.3	0.8 ± 0.3
Intact plasma membrane & acrosome (%)[B]	75.7 ± 3.7[a]	58.8 ± 8.6[b]
Normal morphology (%)[C]	79.0 ± 5.7	71.2 ± 15.4

[A]Motile sperm agglutination rating: 0=none, 5=100% of motile cells agglutinated. [B]Determined by staining with propidium iodide & FITC-PNA. [C]Determined from plasma membrane intact cells only. [a,b]Values with a different superscript within the same row are different (*P* < 0.05).

Table 3. Characteristics of sorted and non-sorted African elephant sperm (n = 3 ejaculates from 1 male) undergoing liquid storage for 24 h at 5°C (n=9 replicates per treatment group).

Parameter	*Sorted Spermatozoa (15 x 10^6 sperm/ml)*	*Non-sorted Spermatozoa Standard Concentration (100 x 10^6 sperm/ml)*	*Non-sorted Spermatozoa Low Concentration (15 x 10^6 sperm/ml)*
0 h Post-Sorting &/or Storage at 5 ºC			
Total motility (%)	82.0 ± 6.1[a]	61.8 ± 9.0[b]	61.3 ± 5.5[b]
Progressive motility (%)	50.0 ± 9.1[a]	30.5 ± 9.1[b]	34.8 ± 8.5[b]
Velocity (average pathway velocity, µm/s)	117.0 ± 3.6[a]	106.8 ± 6.9[b]	112.9 ± 7.3[b]
Agglutination rating (0-5)[b]	0.6 ± 0.4[a]	0.9 ± 0.2[a,b]	1.1 ± 0.2[b]
Intact plasma membrane & acrosome (%)[B]	88.3 ± 5.6[a]	72.2 ± 11.9[b]	74.3 ± 6.7[b]
Normal morphology (%)[C]	86.2 ± 4.8[a]	75.5 ± 9.2[b]	76.0 ± 6.9[b]
24 h Post-sorting &/or Storage at 5 ºC			
Total motility (%)	63.8 ± 6.1[a]	35.9 ± 14.2[b]	6.7 ± 2.3[c]
Progressive motility (%)	46.0 ± 7.3[a]	9.5 ± 7.3[b]	1.7 ± 0.5[c]
Velocity (average pathway velocity, µm/s)	100.2 ± 23.2[a]	62.8 ± 20.1[b]	41.2 ± 6.8[c]
Agglutination rating (0-5)[A]	0.8 ± 0.3	1.0 ± 0.0	0.5 ± 0.0
Intact plasma membrane & acrosome (%)[B]	85.6 ± 3.7[a]	72.4 ± 4.9[b]	69.0 ± 9.3[b]
Normal morphology (%)[C]	82.0 ± 6.7[a]	62.0 ± 2.0[b]	66.0 ± 2.0[b]

[A]Motile sperm agglutination rating: 0=none, 5=100% of motile cells agglutinated. [B]Determined by staining with propidium iodide & FITC-PNA. [C]Determined from plasma membrane intact cells only. [a,b,c]Values with a different superscript within the same row are different (*P* < 0.05).

Rhinoceros

Csi sex-sorted spermatozoa derived from viable electroejaculates displayed *in vitro* characteristics that were maintained throughout a 36 h chilled storage period (Table 4). *In vitro* quality of Run and Dbi spermatozoa from viable samples was acceptable post-transport (e.g.

total motility: Dbi: 54-85%; Run: 81-88%) but further trials are required to improve initial semen quality (Dbi) and optimize diluents for X-Y resolution (Run).

Table 4. Characteristics of sorted and non-sorted white rhinoceros spermatozoa (n = 2 males, 1 ejaculate/male) undergoing liquid storage for 36 h at 5°C (n=6 replicates per treatment group).

Parameter	*Sorted Spermatozoa (19 x 10^6 sperm/ml)*	*Non-sorted Spermatozoa (19 x 10^6 sperm/ml)*
0 h Post-Sorting &/or Storage at 5ºC		
Total motility (%)	83.5 ± 8.8	86.5 ± 2.3
Progressive motility (%)	63.3 ± 9.9	50.7 ± 5.7
Velocity (average pathway velocity, µm/s)	98.5 ± 10.1	113.0 ± 14.7
Agglutination rating (0-5)[A]	0.0 ± 0.0	0.0 ± 0.0
Intact plasma membrane & acrosome (%)[B]	88.0 ± 4.3	89.8 ± 1.7
18 h Post-sorting &/or Storage at 5ºC		
Total motility (%)	69.3 ± 13.2	81.0 ± 4.5
Progressive motility (%)	61.3 ± 10.7	48.0 ± 9.2
Velocity (average pathway velocity, µm/s)	83.2 ± 3.7	96.0 ± 9.2
Agglutination rating (0-5)[A]	0.0 ± 0.0	0.0 ± 0.0
Intact plasma membrane & acrosome (%)[B]	85.5 ± 10.3	84.7 ± 6.9
36 h Post-sorting &/or Storage at 5ºC		
Total motility (%)	41.5 ± 7.3	73.0 ± 7.7
Progressive motility (%)	33.5 ± 3.1	45.7 ± 19.8
Velocity (average pathway velocity, µm/s)	74.7 ± 10.0	79.0 ± 8.8
Agglutination rating (0-5)[A]	0.0 ± 0.0	0.0 ± 0.0
Intact plasma membrane & acrosome (%)[B]	81.8 ± 9.7	82.8 ± 3.4

[A]Motile sperm agglutination rating: 0=none, 5=100% of motile cells agglutinated. [B]Determined by staining with propidium iodide & FITC-PNA. *(Data are needed from an additional male to provide a minimum of 9 replicates per treatment group and permit statistical analyses).*

In vitro quality of sex-sorted spermatozoa after long-term preservation

Rhinoceros

Csi sex-sorted spermatozoa derived from viable electroejaculates displayed *in vitro* characteristics that were maintained after sorting, and after cryopreservation using directional freezing technology (Table 5).

Table 5. Characteristics of sorted and non-sorted white rhinoceros spermatozoa (n = 2 males, 1 ejaculate/male) undergoing directional freezing and thawing (n=6 replicates per treatment group).

Parameter	*Sorted Spermatozoa*	*Non-sorted Spermatozoa*
0 h Post-thaw		
Total motility (%)	45.2 ± 6.7	47.8 ± 10.1
Progressive motility (%)	34.0 ± 7.3	36.3 ± 7.3
Velocity (average pathway velocity, µm/s)	62.5 ± 5.1	71.5 ± 11.4
Agglutination rating (0-5)[A]	0.0 ± 0.0	0.0 ± 0.0
Intact plasma membrane & acrosome (%)[B]	58.5 ± 5.8	53.7 ± 6.0
3 h Post-thaw		
Total motility (%)	36.8 ± 8.7	37.3 ± 9.7
Progressive motility (%)	24.2 ± 2.9	27.2 ± 8.3
Velocity (average pathway velocity, µm/s)	57.9 ± 9.4	63.9 ± 12.1
Agglutination rating (0-5)[A]	0.0 ± 0.0	0.0 ± 0.0
Intact plasma membrane & acrosome (%)[B]	56.3 ± 11.5	53.5 ± 3.5

[A]Motile sperm agglutination rating: 0=none, 5=100% of motile cells agglutinated. [B]Determined by staining with propidium iodide & FITC-PNA. *(Data are needed from an additional male to provide a minimum of 9 replicates per treatment group and permit statistical analyses).*

Discussion

Spermatozoa undergoing sorting are exposed to various stressors including nuclear staining, high dilution, exposure to laser light, high pressure and centrifugation. The potential detrimental effect of such processes on sperm function is exacerbated by conditions spermatozoa are exposed to prior to sorting. Elephant and rhinoceros ejaculates containing increased proportions of degenerative spermatozoa (due to presumptive prolonged storage in the male reproductive tract prior to ejaculation), and elevated creatinine concentration (due to urine contamination), displayed reduced *in vitro* sperm quality after sorting. Results of the current study demonstrate that pachyderm ejaculate quality after collection by manual stimulation or EEJ is highly variable, and specific *in vitro* criteria of initial ejaculate quality are required to allow the production of high quality sex-sorted spermatozoa.

The considerable difference in DNA content between X- and Y-spermatozoa for elephants (Ema: 3.4%, Laf: 3.7-3.9%[15,16]) and rhinoceroses (Csi, Dbi: 4.1%[16]; Run: 4.2%[16]) enables effective enrichment of samples for the desired sex. Sorting efficiency, reflected by the sorting rate and the recovery of functional spermatozoa during sorting and post-sorting processes, will vary according to the initial proportion of viable cells, and in response to other factors such as egg yolk concentration during staining/sorting, and operator/instrumental effects. Sex-sorting trials were performed in an Asian elephant with acceptable sorting rates (1945 X-sperm/s) and *in vitro* sperm quality (58% total motility, 32% progressive motility) after sorting and chilled storage for 12 h[6]. In the present study, similar sorting rates were achieved and extensive examination of Ema sperm *in vitro* quality at multiple time-points after sorting and chilled storage revealed that samples should be suitable for AI if inseminated within 24 h after sorting. Results herein also provide new information on the sex-sorting of Laf spermatozoa; samples from this species also displayed high *in vitro* quality after sorting and chilled storage.

For rhinoceroses, low sorting rates (300-700 X-sperm/s) and poor *in vitro* quality (12% total motility at 0 h post-sorting) were previously reported for Csi and Dbi[2]. In the current study, Csi

spermatozoa derived from viable electroejaculates displayed moderate sorting rates (2160 ± 509 X-sperm/s). *In vitro* characteristics of sorted Csi spermatozoa were well-maintained after sorting and 36 h of chilled storage, and after cryopreservation and thawing using directional freezing technology. Collective results support the use of either chilled or frozen-thawed sex-sorted Csi spermatozoa in AI trials. Further research is required in other rhino species to address reduced ejaculate quality after EEJ (Dbi), and X-Y sperm resolution (Run).

The ability to produce elephant and rhinoceros offspring of predetermined sex continues to depend on strategic optimization of each step in the sorting and sperm preservation process. The majority of elephant ejaculates collected during this research were not of suitable quality for sorting as diluted samples exceeded 0.013 mg/ml of creatinine (indicative of urine contamination) and contained more than 15% of spermatozoa with detached heads (indicative of aged cells). In the rhinoceros, the same ejaculate criteria were required for spermatozoa to be capable of tolerating the cumulative stressors of chilled storage, sorting and short- or long-term preservation. Inconsistent ejaculate quality is the greatest challenge to the application of sorting technology and its integration into AI programs for each species. Further research is required in all species to increase the proportion of non-contaminated ejaculates (i.e. free of urine and aged sperm cells) collected after both manual stimulation and EEJ. Such improvements will thereby facilitate the ability to sort and cryopreserve adequate numbers of functional sex-sorted spermatozoa for future use in AI or other assisted fertilization procedures.

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Sperm Sorting and Preservation Technologies for Sex Ratio Modification in the Elephant and Rhinoceros: An Update

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Elephant & Rhinoceros
Sperm Sorting & Preservation Research

Overall goal: Develop Sperm Sorting & Sperm Preservation Methodologies for Sex Predetermination of Offspring using Artificial Insemination

- Correct undesirable sex ratio skews (from natural[1] and artificial insemination[2])
- Maintain maximal **genetic diversity**
- Assist in providing optimal **social environments**
- Increase **population growth rate**
- **Sustainability of *ex situ* populations**

[1]*Roth et al., 2005;* [2]*Saragusty et al., 2009*

Research Objectives

- Establish semen collection & pre-sorting storage methods & initial **ejaculate quality** necessary for production of high quality sex-sorted sperm

- Determine the *in vitro* quality of sex-sorted sperm after **short-term preservation** (chilled storage)

- Examine the *in vitro* quality of sex-sorted sperm after **long-term preservation** (cryopreservation, *Ceratotherium simum simum* only).

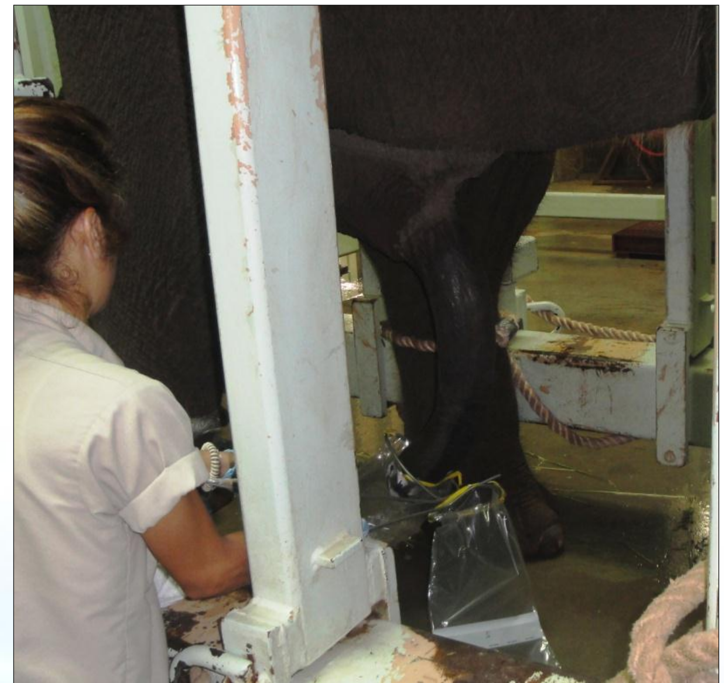
Elephant Research Methods

Animals:

- 8 *Elephas maximus* (aged 11 to 38 yrs)
- 2 *Loxodonta africana* (aged 9 & 28 yrs)

Semen collection & pre-sorting storage:

- Manual stimulation (n = 118)
- Clean-out collections prior to experiments
- Minimize contamination : penis rinsing
- Pre-load sterile collection bags with diluent
- Dilution of semen for overnight transport



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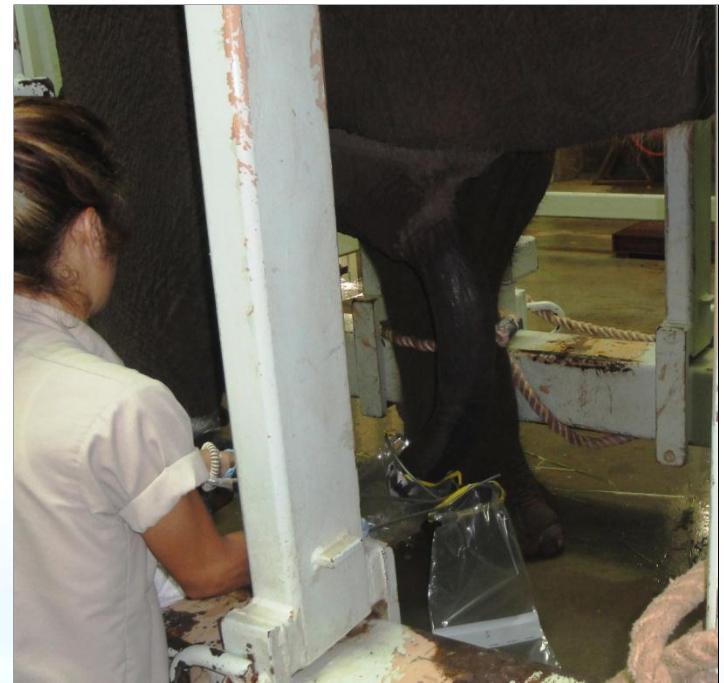

[1]*Schmitt and Hildebrandt, Anim. Reprod. Sci. (1998)*

Rhinoceros Research Methods

Animals:

- 5 *Diceros bicornis michaeli* (7 to 13 yrs)
- 3 *Ceratotherium simum simum* (8 to 11 yrs)
- 3 *Rhinoceros unicornis* (8 to 32 yrs)

Semen collection & pre-sorting storage:

- Electroejaculation (n = 20)
- Encourage urination prior to EEJ
- Minimize contamination : penis rinsing
- Pre-load sterile collection bags with diluent
- Dilution of semen for transport (arrival at lab ~12 h post-collection)

Rhinoceros Research Methods

Animals:

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[1] *Roth et al., JZWM (2005)*

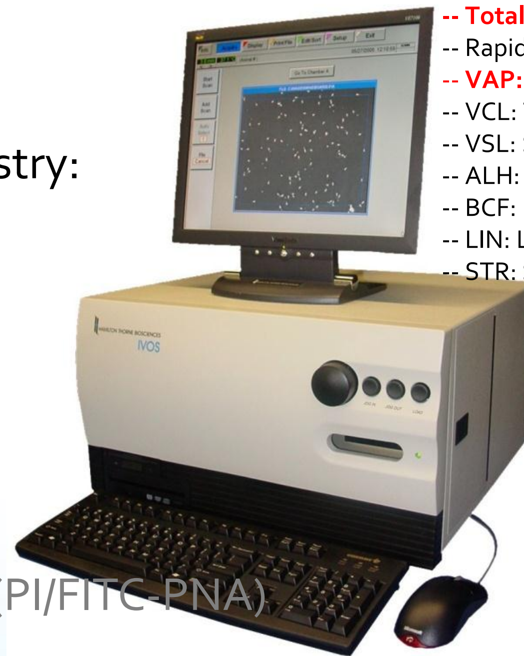
Elephant & Rhino Research Methods

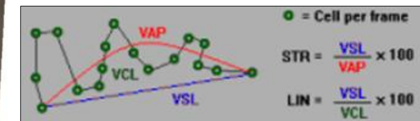
Ejaculate & Sperm Evaluations

- Ejaculate parameters and biochemistry:
  - volume, sperm concentration
  - creatinine, osmolality, pH

- Sperm quality:
  - motility parameters (CASA)
  - agglutination rating
  - viability & acrosome integrity (PI/FITC-PNA)
  - morphology

-- Total motility, Progressive motility
-- Rapid, Medium, Slow and Static Cells
-- VAP: Smoothed/Average Path Velocity (um/sec)
-- VCL: Track Velocity (um/sec)
-- VSL: Straight Line Velocity (um/sec)
-- ALH: Amp of Lateral Head Displacement (um)
-- BCF: Beat Cross Frequency (hertz)
-- LIN: Linearity (ratio of VSL/VCL)
-- STR: Straightness (ratio of VSL/VAP)





Hamilton Thorne

Elephant & Rhino Research Methods

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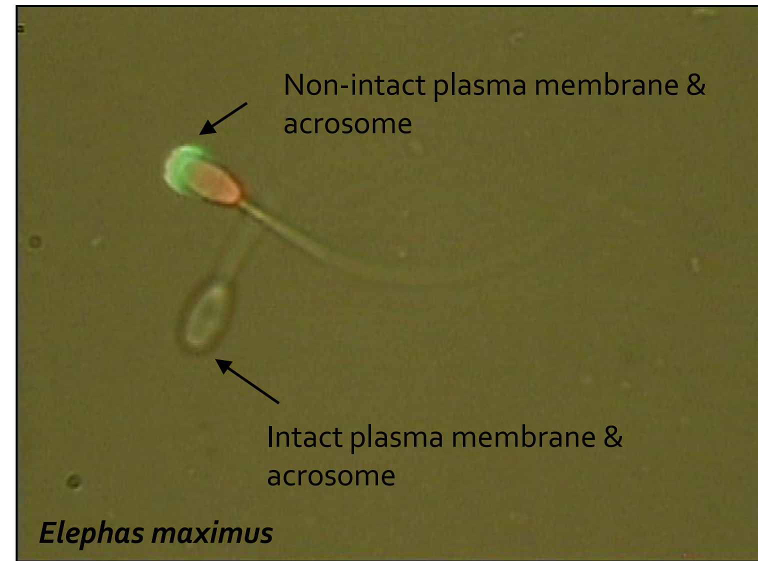
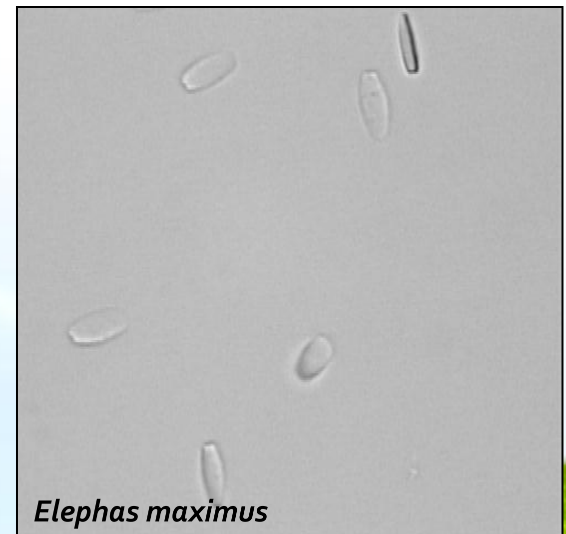
  - volume, sperm concentration
  - creatinine, osmolality, pH
- Sperm quality:
  - motility parameters (CASA)
  - agglutination rating
  - **viability & acrosome integrity (PI/FITC-PNA)**
  - **morphology**



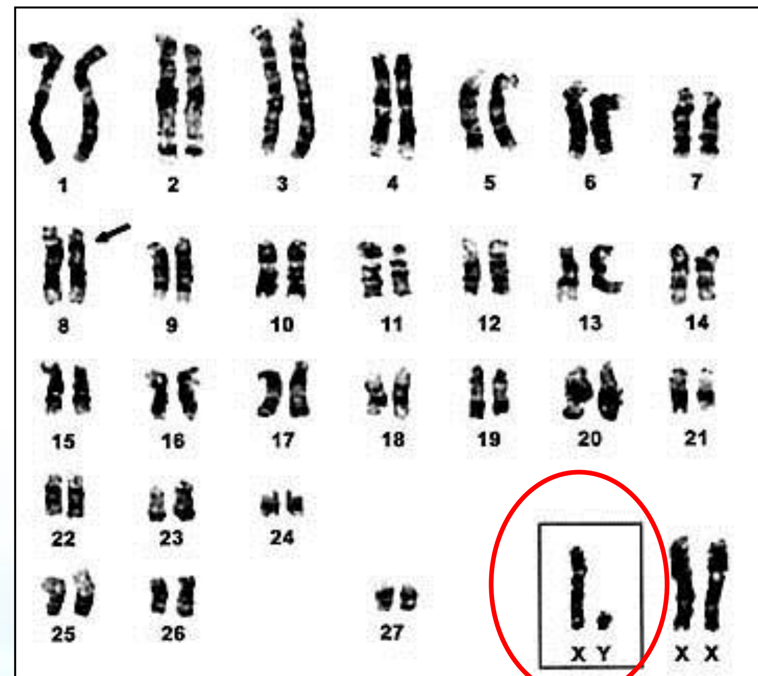


Elephant & Rhino Research Methods

Gorilla	2.7
Orangutan	3.2
Chimpanzee	3.3
Rabbit	3.0
Asian elephant	**3.4%**
Zebra	3.7
Hippo	3.7
Cattle	**3.8**
Elk	3.8
Dog	3.9
African elephant	**3.9%**
Dolphin	4.0
Beluga	4.0
Killer whale	4.0
Marmoset	4.1
Black rhino	**4.1%**
White rhino	**4.1%**
Indian rhino	**4.2%**
Baboon	4.2
Tiger	4.1
Lemur	4.3
Macaque	4.3
Giraffe	4.4
Chinchilla	7.5

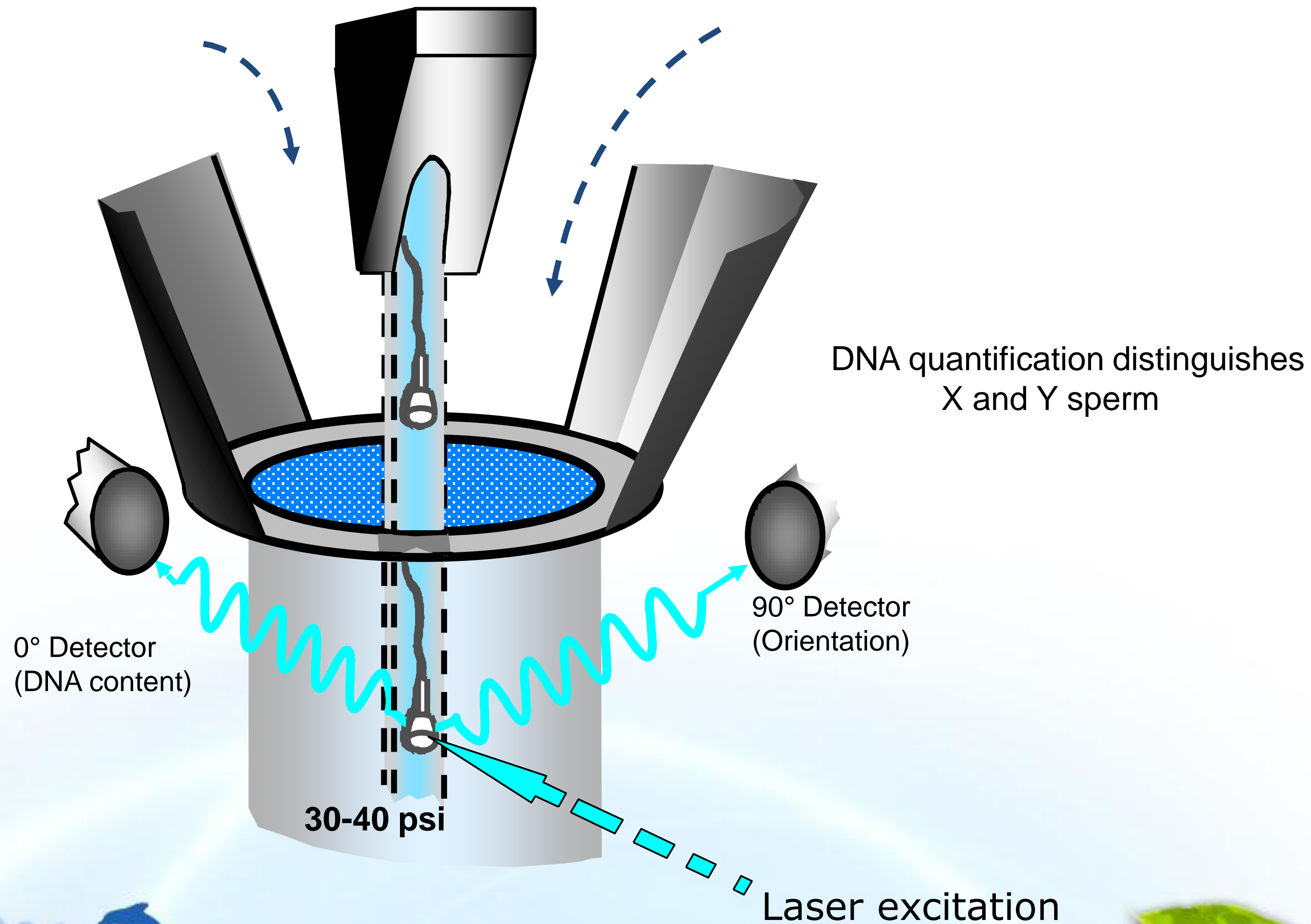
O'Brien et al, Theriogenology (2009)

Basis of Sperm Sorting: Difference in DNA content



African elephant karyotype (Houck et al., Cyto Cell Genet (2001))

Elephant & Rhino Research Methods



Johnson et al., Biol. Reprod. (1989)

Elephant & Rhino Research Methods

Asian Elephant:

256
192
128
64
0

Relative DNA content (forward angle fluorescence)

Live, oriented

Degenerating/dead

0 64 128 192 256

Orientation (Side angle fluorescence)

Y X

Counts

381
285
190
95
0

181 191 201 212 222

Relative DNA content

3.4% X-difference
2200 X-sperm/sec
Accuracy: ≥89%

Elephant & Rhino Research Methods

Asian Elephant:

256
192
128
64
0
Relative DNA content (forward angle fluorescence)

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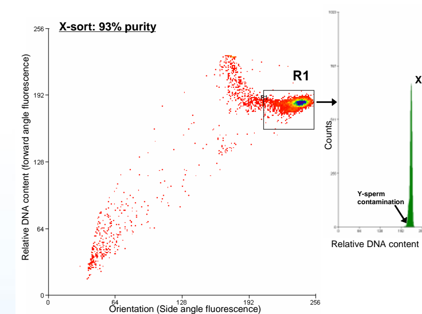


Elephant & Rhino Research Methods

X-sort: 93% purity

Relative DNA content (forward angle fluorescence)

256
192
128
64
0

R1

0 64 128 192 256

Orientation (Side angle fluorescence)

Counts

X

Y-sperm contamination

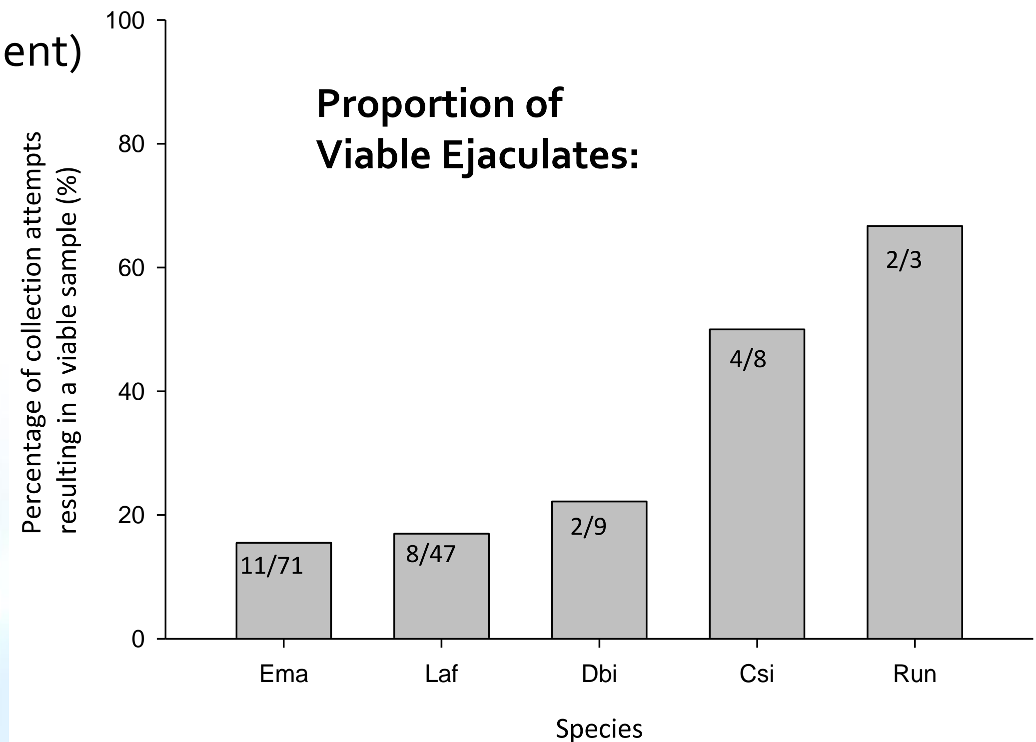
Relative DNA content

3.4% X-difference
2200 X-sperm/sec
Accuracy: ≥89%

Elephant & Rhinoceros Results

Classification of Viable Ejaculates:

- > 70% progressive motility & plasma/acr membrane integrity
- < 0.013 mg creatinine/ml (w/ diluent)
- < 15% detached heads



Elephant & Rhinoceros Results

Post-Sorting Quality of Non-Viable Ejaculates:

- **Elephant:**

  - < 40% progressive motility

  - < 75µm/s average path velocity

- **Rhino**

  - < 40% progressive motility

  - < 50µm/s average path velocity

- **Poor maintenance of sorted sperm quality** during chilled storage/cryopreservation

Elephant & Rhinoceros Results

Optimum Base Diluent

- **Elephant**:

  - Test Yolk buffer (modified from Graham et al., 1972)
  - 10% egg yolk, Tes, Tris, fructose, Na-pyruvate, BSA, antibiotics cocktail (Gent, tylosin, lincomycin, spectinomycin)

- **Rhino**:
  - EQ (modified from Martin et al., 1979; O'Brien & Roth, 2000)
  - 10% egg yolk, citrate, $NaHCO_3$, lactose, glucose, EDTA, Equex, Pen-strep

Staining and sheath media were egg yolk free (≤ 5% EY @staining)
Cryopreservation media contained 20% egg yolk

Chilled Storage Results: Elephant

Sorted vs Non-sorted

Motility, Viability, Acrosome integrity (%)
VAP (µm/s)

0h

Sorted
Non-sorted

120
110
100
90
80
70
60
50
40
30
20
10
0

Total Motility | Prog Motility | Viable&Acr Intact | VAP

24h

Sorted
Non-sorted

120
110
100
90
80
70
60
50
40
30
20
10
0

Total Motility | Prog Motility | Viable&Acr Intact | VAP

(n=9 reps/trt)

Ema: > 65% parameters maintained over 24 h post-sorting except prog. motility (31%)

Chilled Storage Results: Elephant

Sorted vs Non-sorted

0h

Motility, Viability, Acrosome integrity (%)
VAP (µm/s)

120
110
100
90
80
70
60
50
40
30
20
10
0

Sorted
Non-sorted

*

Total Motility
Prog Motility
Viable&Acr Intact
VAP

24h

120
110
100
90
80
70
60
50
40
30
20
10
0

Sorted
Non-sorted

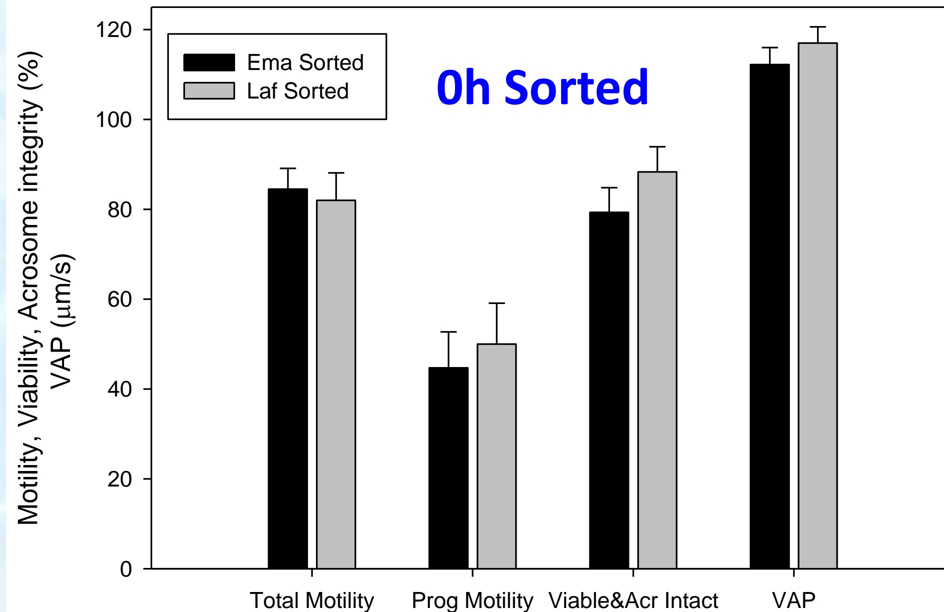
*

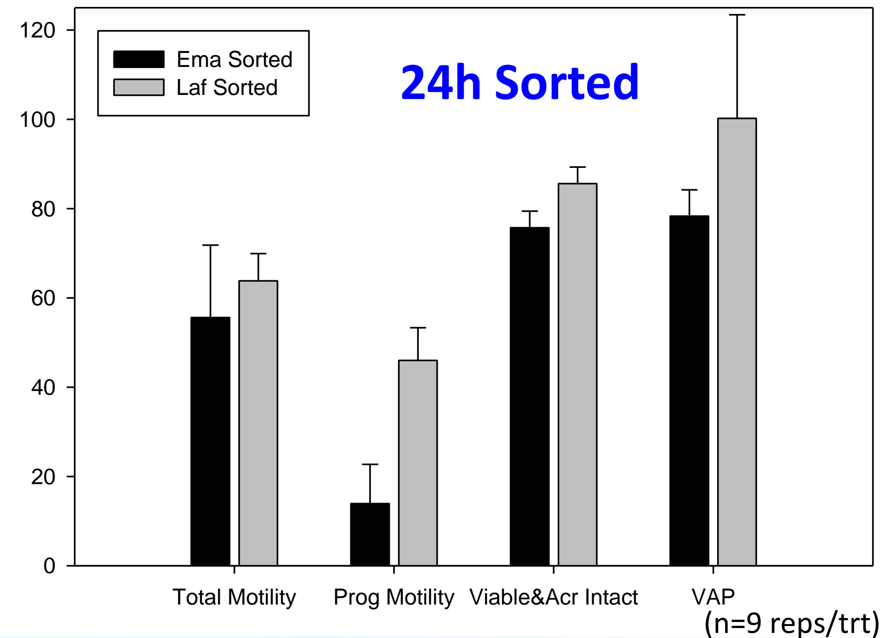
Total Motility
Prog Motility
Viable&Acr Intact
VAP

Ema: > 65% parameters maintained over 24 h post-sorting except prog. motility (31%)

Chilled Storage Results: Elephant

Asian vs African





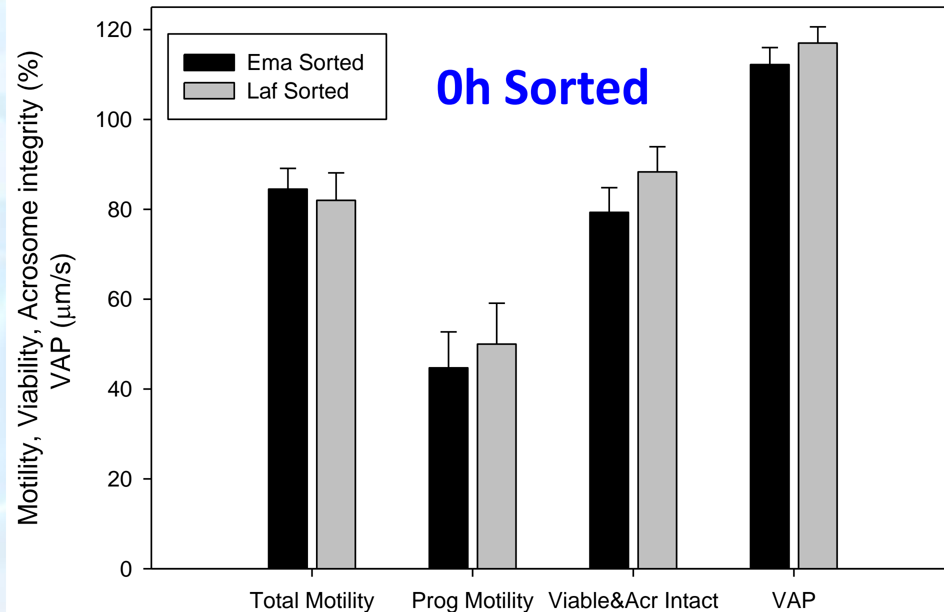
(n=9 reps/trt)

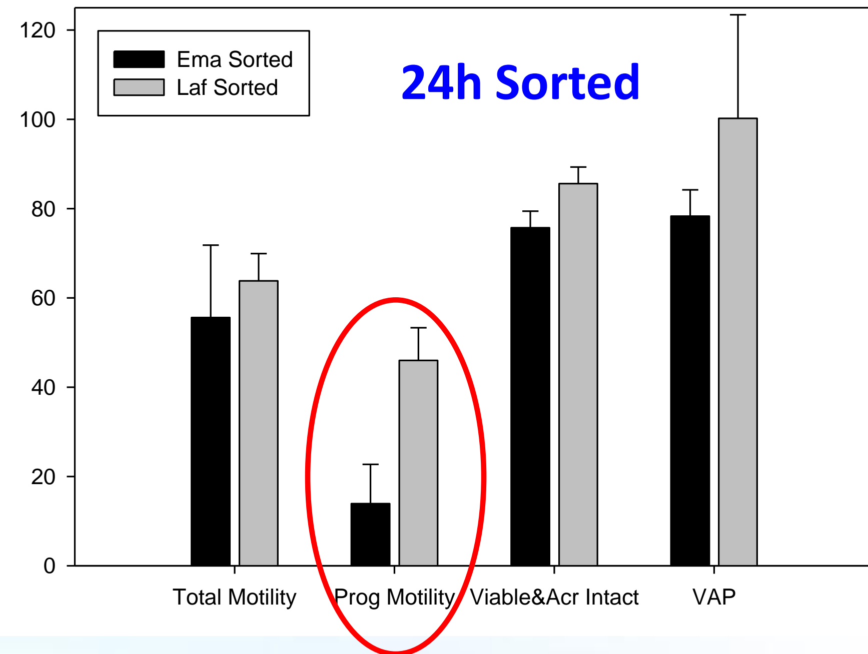
Ema: > 65% of 0h parameters maintained over 24 h except prog. motility (31%)

Laf: > 77% of all 0h parameters maintained (92% prog. motility)

Chilled Storage Results: Elephant

Asian vs African



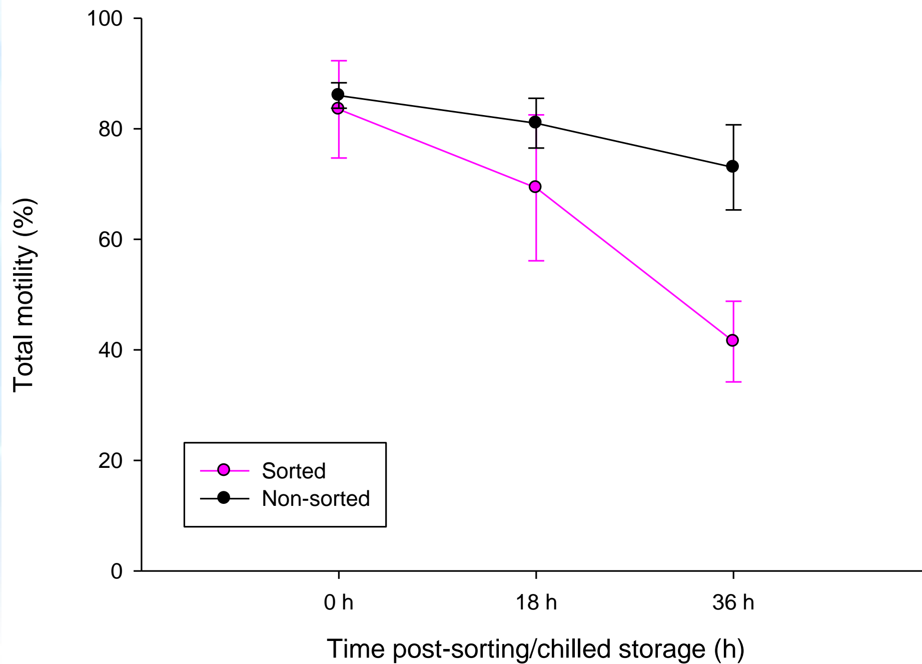


Ema: > 65% of 0h parameters maintained over 24 h except prog. motility (31%)

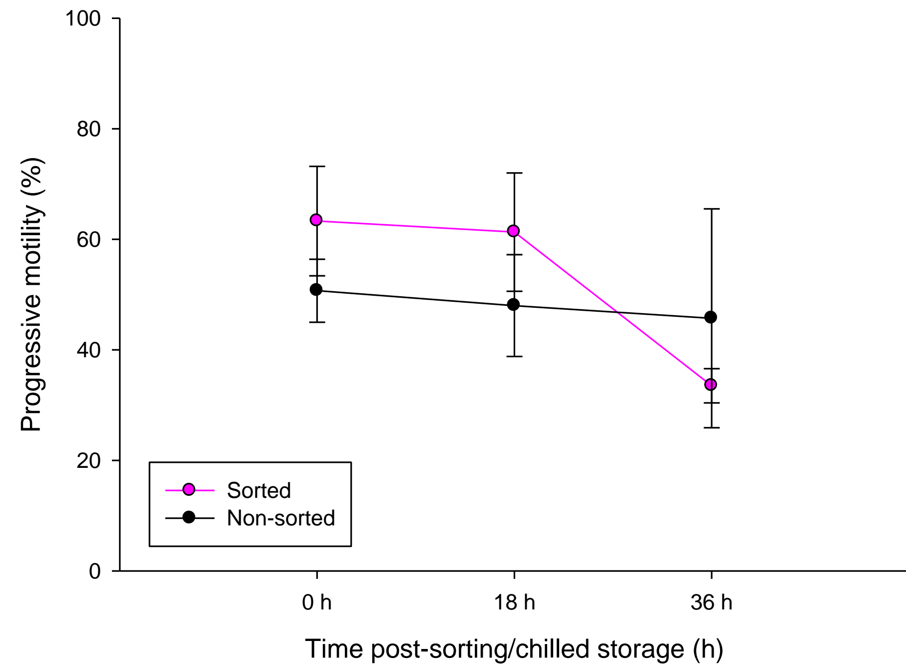
Laf: > 77% of all 0h parameters maintained (92% prog. motility)

Chilled Storage Results: White Rhino

Total motility:



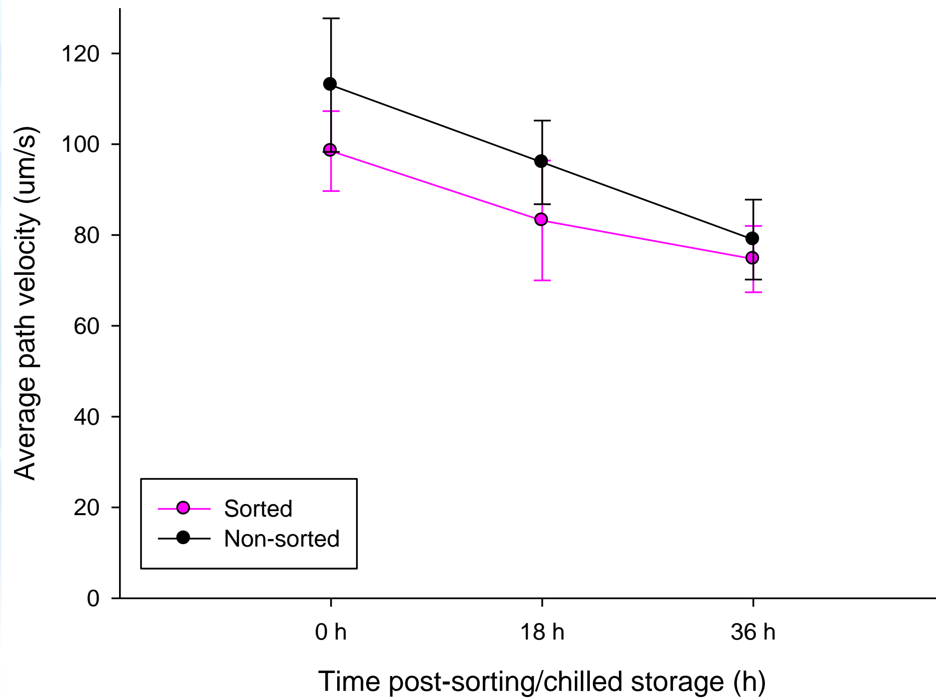
Progressive motility:

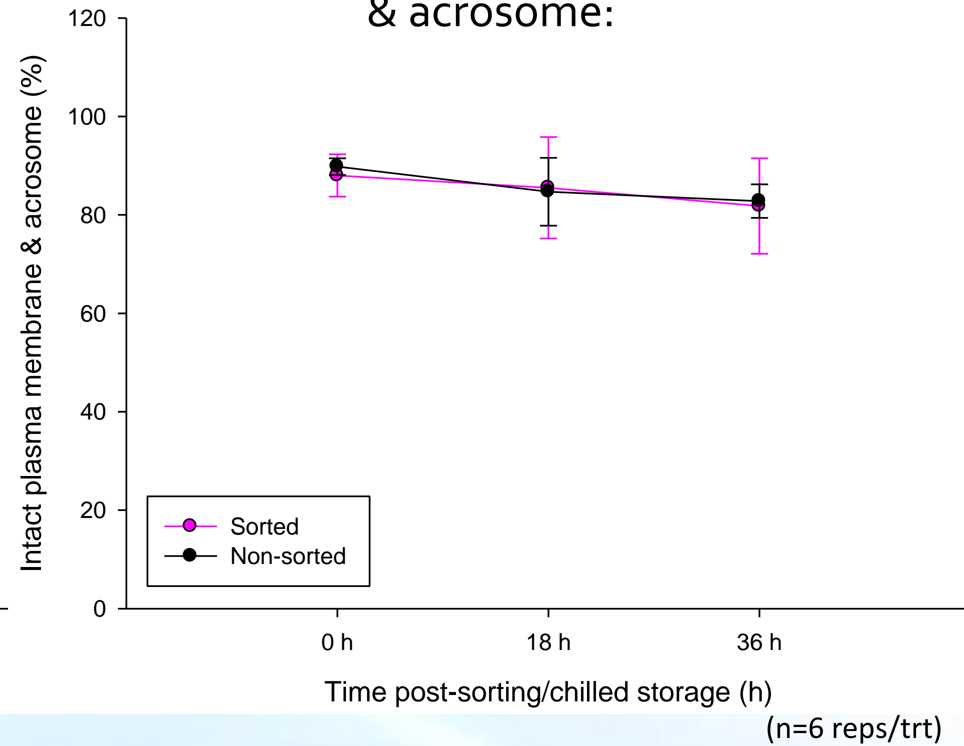


(n=6 reps/trt)

50-53% of 0h motility was maintained over 36 h

Chilled Storage Results: White Rhino





(n=6 reps/trt)

76% of 0h velocity & 93% membrane integrity maintained

Cryopreservation Results: White Rhino

Cryopreservation of Sorted and Non-sorted Sperm: Directional freezing

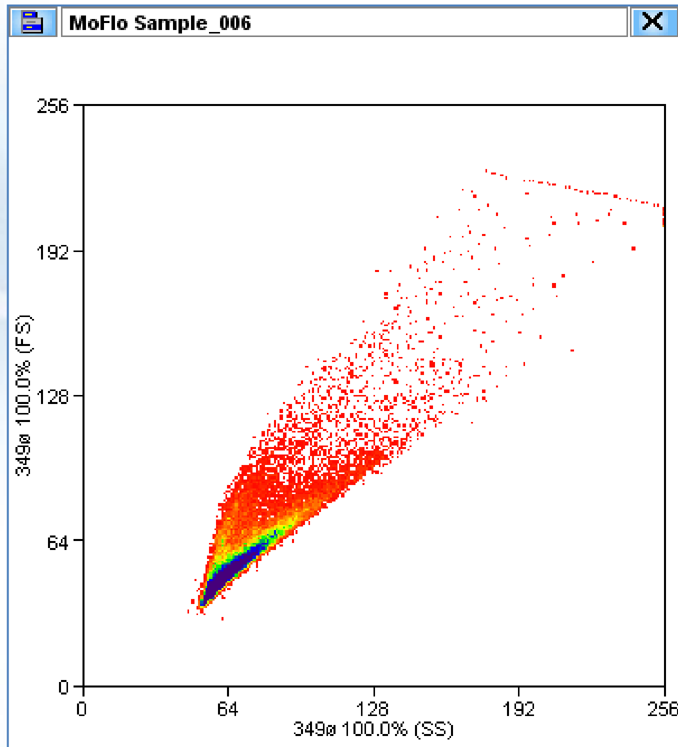
Parameter	*Sorted Sperm*	*Non-sorted Sperm*
0 h Post-thaw		
Total motility	45.2 ± 6.7	47.8 ± 10.1
Progressive motility	34.0 ± 7.3	36.3 ± 7.3
Velocity (average pathway velocity, µm/s)	62.5 ± 5.1	71.5 ± 11.4
Agglutination rating (0-5)[A]	0.0 ± 0.0	0.0 ± 0.0
Plasma membrane & acrosome intact (%)[B]	58.5 ± 5.8	53.7 ± 6.0
3 h Post-thaw		
Total motility	36.8 ± 8.7	37.3 ± 9.7
Progressive motility	24.2 ± 2.9	27.2 ± 8.3
Velocity (average pathway velocity, µm/s)	57.9 ± 9.4	63.9 ± 12.1
Agglutination rating (0-5)[A]	0.0 ± 0.0	0.0 ± 0.0
Plasma membrane & acrosome intact (%)[B]	56.3 ± 11.5	53.5 ± 3.5

(n=6 reps/trt)

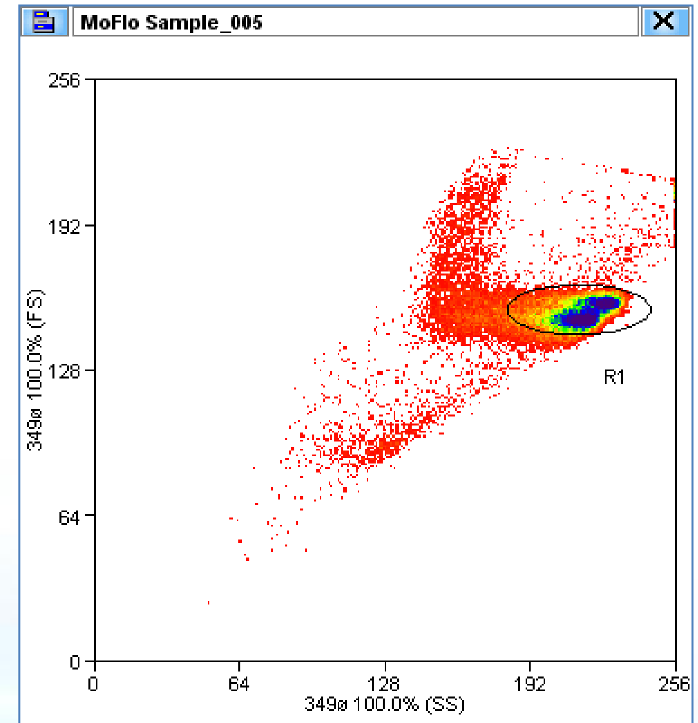
51-53% of pre-freeze motility maintained after thawing
62-65% of pre-freeze velocity and membrane integrity

Sorting Results: Indian Rhino

10% Egg yolk in transport diluent:



Egg yolk removed (DGC):



Indian: Poor quality post-sorting in absence of EY during staining
(Black: further viable samples req' for liquid storage/cryo trials)

Sorting Parameters

Species	Sex-Sorted Sperm Production (million/hr)	Progressively motile sperm per AI ($x10^6$)	Sorting Time Fresh AI (h) **(1 Sorter)**	Sorting Time Frozen AI (h) **(1 Sorter)**	Practical Application
Cattle	15	1	0.082 h (~5 min)	0.16 h (~10 min)	AI
Dolphin	15	50	4.1 h	8.2 h	AI
Elephant	8	*1000*	218 h (18 d)	436 h (36 d)	AI
Rhinoceros	8	620	135 h (11 d)	270 h (23 d)	AI

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Summary

- **Characterization of ejaculate quality necessary for sorting**
  - Urine contamination, aged cells
  - Detrimental effects are not evident at collection (urine)

- **Sorted sperm quality**
  - Maintained during chilled storage (Ema[1], Laf, Csi)
  - Maintained during freeze-thawing (Csi)

- **Efficiency of sorting**
  - ≤20% of initial population reside in X-sample
  - Reduced homogeneity of H33342 staining from egg yolk

[1] *Hermes et al., Anim. Reprod. Sci.(2009)*

Future Directions

- **All Species**
  - Urine contamination, aged cells
    - Urination pre-EEJ/manual collection
    - Rhino: Catheterize bladder pre-EEJ
- **Elephant**
  - Sorting/banking of frozen AI dose
    - Cryopres. Trials; Sorting/banking within 24 h of semen collection.
- Rhino
  - Black: Poor sperm quality
    - EEJ of breeding males, Nutritional Tx.
  - Indian: best sperm quality but poor X-Y resolution
    - Non-egg yolk diluents
  - White: Good post-thaw quality but low sperm #/ejaculate
    - Low dose, deep intrauterine AI (100 million PMS)

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Acknowledgments

<u>**All Curatorial, Keeper & Veterinary staff at Collaborating Institutions:**</u>

Albuquerque Biological Park (R. Saiers, A. Saunders)
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Dickerson Park Zoo (J. Bradford)
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Fort Worth Zoo (R. Surratt, J. Barr)
Indianapolis Zoo (D. Hagan, D. Olson)
Montgomery Zoo
Oregon Zoo (M. Finnegan)
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Taronga Zoo (G. Miller)
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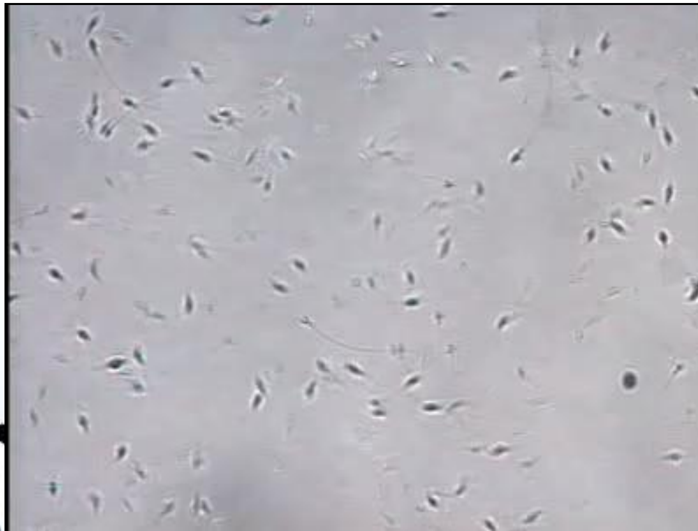
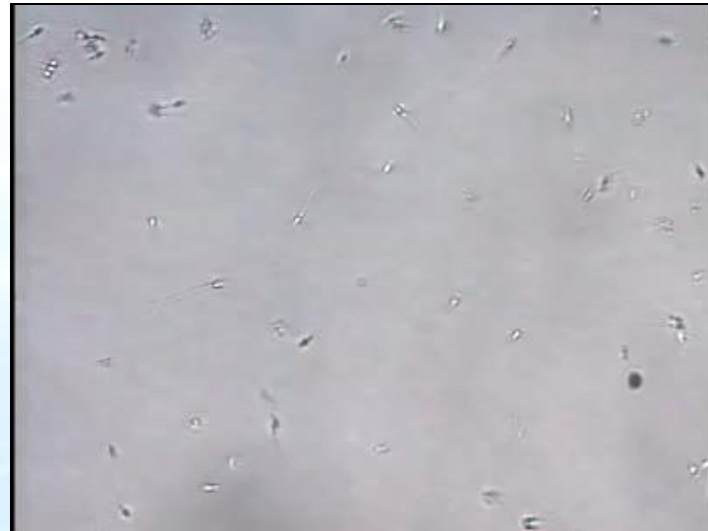
Genome Banking: Gamete Collection & Preservation

Semen Collection Training

- Goal:
  - Collection of concentrated, non-contaminated ejaculates on a consistent basis
- **Challenges:**
  - **Contamination (saltwater, lubricant, urine, aged sperm)**
  - Provision of adequate stimulation to obtain a normal ejaculatory response
  - Social /behavioral suppression of reproduction
  - Seasonal effects on reproduction





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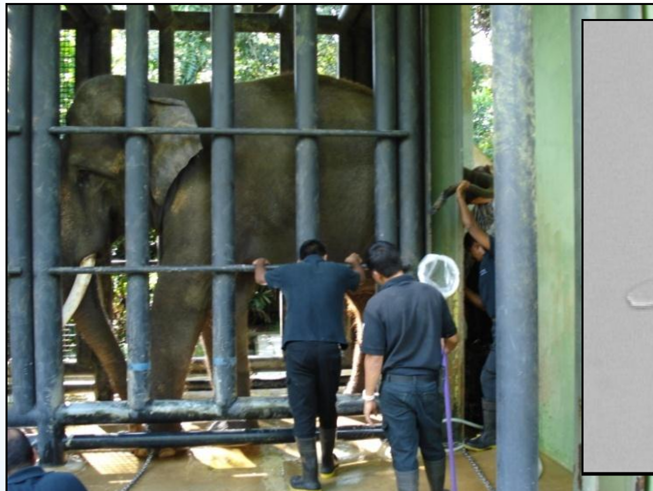
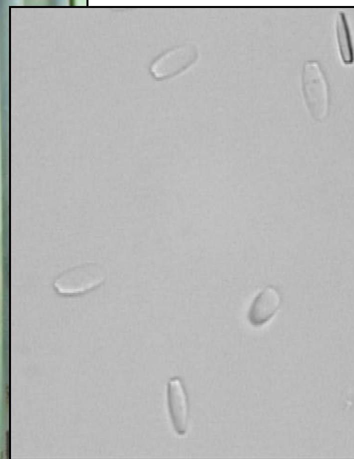
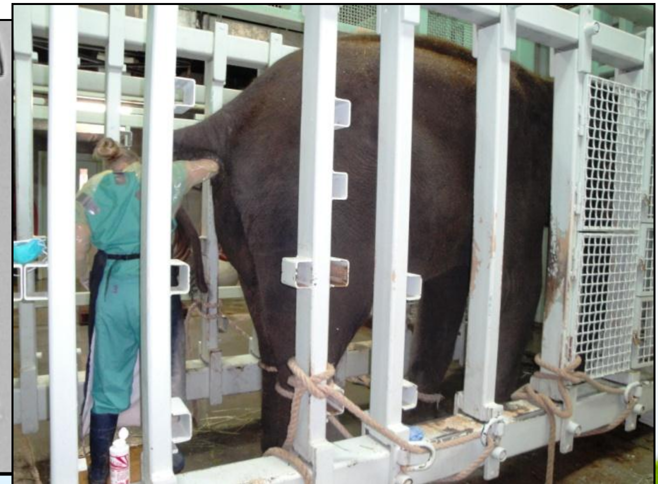


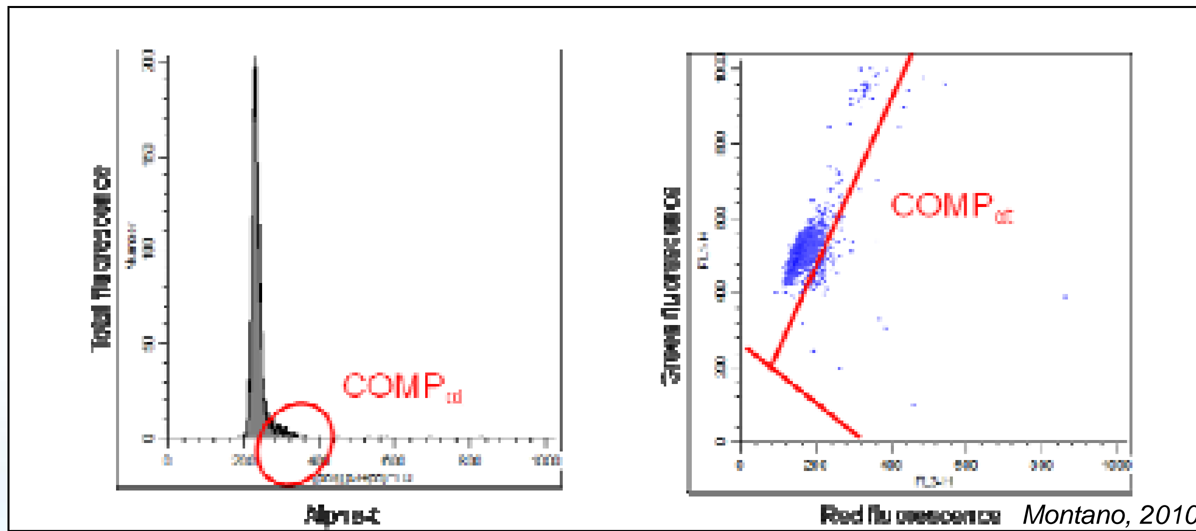




Semen and Sperm Quality Analysis

DNA quality: Sperm Chromatin Stability Assay (SCSA)

- Susceptibility of sperm DNA to denaturation after a exposure to low pH
- Acridine orange staining (Evenson method; TAMU)
- Identifies double stranded (normal) and single stranded (abnormal) DNA
- Master's Research Project in *Tursiops truncatus* (G. Montano)

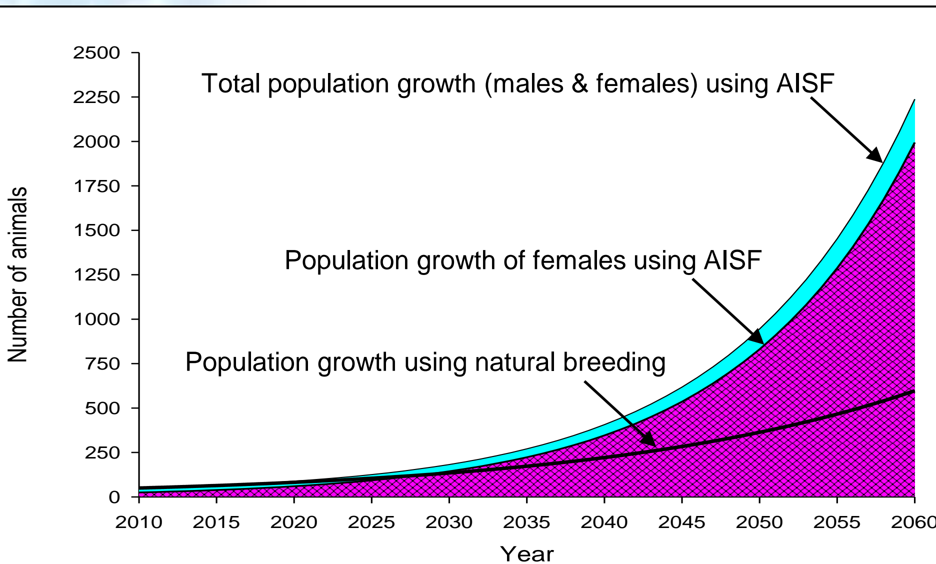


Montano, 2010

Reproductive Research Application: Species Management & Conservation

Practical Application

- Routine semen collection & sorting
- Genome banking: non-sorted & sorted sperm
- Population management using sorted sperm AI & natural mating to achieve sex ratio bias (80% females)
- Standard genetic management; maintain genetic diversity



O'Brien and Robeck, Int. J. Comp. Psy. (2010)

- **Social management:**
  Socially cohesive groups (female-dominated)
- **Resource management**:
  Improved use of limited enclosure space
- **Endangered species management:**
  Correct unwanted sex ratio skews
  Population growth increases at a faster rate

Sperm Preservation

Sperm Storage Longevity

Frozen semen storage time (yrs)	No. ewes inseminated	Pregnancy/lambing rate (%)
3	172	91 (53)
5	70	37 (53)
7	143	74 (52)
11	159	88 (55)
16	193	119 (62)
27	205	124 (62)

Salamon and Maxwell (2000) Anim. Reprod. Sci.

Liquid nitrogen storage tanks: LN levels must be regularly monitored