Sperm rescue in wild African elephants

Article *in* Reproduction Fertility and Development · March 2015

DOI: 10.1071/RD14378 · Source: PubMed

7 authors, including:

Joseph Saragusty
Università degli Studi di Teramo
86 PUBLICATIONS **1,808** CITATIONS
SEE PROFILE

Maria Teresa Prieto Pablos
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
33 PUBLICATIONS **522** CITATIONS
SEE PROFILE

Alexandre Courtiol
Leibniz Institute for Zoo and Wildlife Research
70 PUBLICATIONS **1,858** CITATIONS
SEE PROFILE

Romain Potier
FauneVet
18 PUBLICATIONS **130** CITATIONS
SEE PROFILE

CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD14378

Sperm rescue in wild African elephants

Joseph Saragusty[A,D], *María Teresa Prieto*[A], *Alexandre Courtiol*[A], *Romain Potier*[B,C], *Frank Göritz*[A], *Thomas B. Hildebrandt*[A] *and Robert Hermes*[A]

[A]Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Straße 17, D-10315 Berlin, Germany.
[B]ZooParc de Beauval, 41110 Saint Aignan s/ Cher, France.
[C]Present address: FauneVet, CHV Atlantia, 22 rue René Viviani, 44200 Nantes, France.
[D]Corresponding author. Email: saragusty@izw-berlin.de

Abstract. This study investigated ways of improving the usefulness of ~1700 mL of poor-quality frozen semen collected from wild African elephant (*Loxodonta africana*) bulls. Ten semen samples from six bulls, frozen with 5% glycerol in Berliner cryomedium, with or without prior removal of the seminal plasma by centrifugation, were tested. All samples were subjected to the following density-gradient centrifugation treatments: no centrifugation (control), sham centrifugation, Percoll, OptiPrep, Isolate and PureSperm. Sample evaluation included motility, concentration, viability, acrosome integrity and normal morphology after thawing and after gradient centrifugation. Motility was also evaluated 3 h after thawing. While all treatments were similar to the Control in acrosome integrity and normal morphology, significant differences were noted in concentration, viability and motility. Samples treated by Percoll showed the best motility, which was maintained unchanged over 3 h of incubation (37°C). Correlations between manual and automated evaluations of concentration were high (cytometer; rho = 0.92), but were lower for viability (cytometer; rho = 0.57) and motility (computer-aided sperm analysis; rho = 0.66). By performing density centrifugation, the quality of these sperm samples may be improved to a level suitable for artificial insemination in elephants. Although a sizeable proportion of cells are lost in the process, combining samples may still allow for multiple inseminations.

Additional keywords: conservation, cryopreservation, directional freezing, endangered, spermatozoa.

Received 6 October 2014, accepted 2 February 2015, published online 13 March 2015

Introduction

In human medicine and, to some degree, also in domestic and laboratory species, density centrifugation is being used to separate the viable and possibly also morphologically-normal spermatozoa from the rest of the sample. The technique has been in use for several decades and a variety of media and procedures have been developed for this purpose (Van der Zwalmen *et al.* 1991; Claassens *et al.* 1998; Mousset-Siméon *et al.* 2004; Colleoni *et al.* 2011). Utilisation of density centrifugation for spermatozoa collected from wildlife species is far less frequent (Montano *et al.* 2011; Nicolas *et al.* 2012). Density-gradient centrifugation bears obvious advantages when it comes to processing semen from wildlife species. Samples collected from wild animals can often have low motility or a high degree of morphologically-abnormal spermatozoa (Crosier *et al.* 2006; Pukazhenthi *et al.* 2006; Kiso *et al.* 2012). As opportunities to collect semen from wildlife, particularly from endangered or genetically-valuable individuals, are rare, one would not want to waste such opportunities due to poor quality of the samples collected. Even when the collected sample is of good quality, the freezing process is bound to cause some damage to the cells. Furthermore, in the majority of wildlife species semen cryopreservation protocols have not yet been developed. In the process of developing such protocols, a large number of samples are bound to fare poorly. It would be a great waste to discard all these genetically-valuable samples just because their initial quality was poor or because they did not respond well to the cryopreservation process.

The elephant captive population is not self-sustaining due to a wide variety of reasons including small numbers of males in the population, absence of males from many zoos, inappropriate historical reproductive management, poor reproductive health and, in many females, lack of or irregular oestrous cyclicity (Saragusty 2012). For completely different reasons, largely anthropogenic in essence, wild elephant populations over most of their habitat are also decreasing, putting small and isolated populations at risk of extinction (Leimgruber *et al.* 2008; Bouché *et al.* 2011). Importation of both Asian and African elephants from the wild to rejuvenate the captive population was practically brought to a halt when the Convention on

International Trade in Endangered Species of Wild Fauna and Flora (CITES) was enacted in 1976. This puts the captive populations as well as small, isolated populations in the wild at risk of inbreeding and its consequential impaired fitness. For example, an evaluation of the captive African elephant population in North America found an average heterozygosity of 0.53, seemingly lower than the level reported for wild African elephants (0.64; Lei *et al.* 2008). Recently we demonstrated that an alternative way is available (Hildebrandt *et al.* 2012; Hermes *et al.* 2013). By collecting semen from bulls in the wild it is possible to introduce their genetic material into the captive population without removing the animals from their natural habitat. Thus far two elephants (a female and a male) were born and there is a third ongoing pregnancy in a European zoo following artificial insemination with semen collected from wild bulls in South Africa, frozen and shipped to Europe for use in the artificial insemination program. In the process of refining the African elephant semen cryopreservation technique, several alternative protocols were tested. Some of them resulted in very poor post-thaw quality (Hermes *et al.* 2013). We thus found ourselves with ~1700 mL of valuable frozen semen from wild African elephant bulls with quality too poor to be used in artificial insemination programs, exhibiting far less than the minimum acceptable motility level of 30%. The aim of this study was to find ways of improving the quality of the thawed samples so that it will still be possible to use them effectively for artificial insemination. To do that, four different two-layer density-gradient centrifugation media were compared, aiming to remove dead, immotile and possibly also morphologically-abnormal spermatozoa so as to enrich the remaining sample with motile and morphologically-normal spermatozoa. A second objective was to evaluate an automated image cytometer for the assessment of sperm concentration and viability.

Materials and methods

Ethics statement

The described experiments are all laboratory procedures that do not require ethical committee approval.

Semen samples

Semen samples from six African savannah elephant (*Loxodonta africana*) bulls, collected and frozen in the Republic of South Africa and shipped to Europe, were tested. A total of 10 samples were evaluated. Three bulls provided one sample each, two bulls provided two samples each and one bull provided three samples. All samples were initially frozen in Berliner cryomedium extender (see below) containing 5% glycerol. Four of the samples were frozen with the seminal plasma (no centrifugation) and in the other six samples the seminal plasma was removed by centrifugation before the spermatozoa were suspended in freezing extender. All samples were frozen in 8-mL Hollow-Tubes (IMT Ltd, Ness Ziona, Israel) using the directional freezing technique (Hermes *et al.* 2013). Samples were stored under liquid nitrogen for at least 3 years before the experiments. For thawing, samples were held in the air at room temperature (~22°C) for 90 s and then plunged into a water bath (37°C) with a dedicated thawing device (IMT Ltd) for 60 s. The starting point of this study was the quality of the samples upon thawing so sample source and mode of handling before freezing were deemed irrelevant with respect to the objectives of this study. Hereafter, when referring to sperm samples, we mean the 10 samples used for this study.

Gradient centrifugation treatments

After thawing all sperm samples were split into six aliquots that were assigned to one of the following treatments:

(1) No-centrifugation control: thawed aliquot was kept in the water bath at 37°C.
(2) Sham centrifugation: 1 mL of thawed sample was placed in a 15-mL conical centrifugation tube.
(3) Percoll gradient: 1 mL of thawed sample was placed in a 15-mL conical centrifugation tube above a column composed of 2 mL of 45% Percoll (Biochrom AG, Berlin, Germany) over 2 mL of 90% Percoll.
(4) Isolate gradient: 1 mL of thawed sample was placed in a 15-mL conical centrifugation tube above a column composed of 2 mL of 50% Isolate (Irvine Scientific, Santa Ana, CA, USA) over 2 mL of 90% Isolate.
(5) OptiPrep gradient: 1 mL of thawed sample was mixed with 0.67 mL of 60% iodixanol (OptiPrep; Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) and then placed in a 15-mL conical centrifugation tube under an OptiPrep column composed of 2 mL of 20% iodixanol over 2 mL of 40% iodixanol.
(6) PureSperm gradient: 1 mL of thawed sample was placed in a 15-mL conical centrifugation tube above a column composed of 2 mL of 40% PureSperm (Nidacon Laboratories AB, Goteborg, Sweden) over 2 mL of 80% PureSperm.

All media were pre-warmed to 37°C. Samples of the sham centrifugation and all density-gradient groups were centrifuged at 400*g* for 30 min at room temperature in a tilting centrifuge without breaks. All pellets were re-suspended in 1 mL of Berliner cryomedium (BC) basic solution, which was modified for elephants (Saragusty *et al.* 2009). The BC basic solution (Blottner 1998) is composed of 2.41% (w/v) TES, 0.58% (w/v) Tris, 0.1% (w/v) fructose, 5.5% (w/v) lactose, 15.6% (v/v) egg yolk and 20 IU mL^{-1} α-tocopherol.

Sperm evaluation

Samples were evaluated for motility, concentration, viability, acrosome integrity and morphology after thawing and after centrifugation. Motility was also assessed 3 h after thawing (just over 2 h after centrifugation) and incubation at 37°C. Total recovered volume was assessed using a pipette and sperm concentration was estimated using a Neubauer haemocytometer. Concentration was also estimated using an image cytometer (see below).

Motility was evaluated subjectively by phase-contrast microscopy at 100× and 200× magnification. The same experienced spermatologist evaluated all samples. In parallel, post-thaw samples and sperm suspensions obtained after density-gradient centrifugation were also analysed using a computer-aided sperm analysis (CASA) system. Samples (5 μL) were loaded onto

pre-warmed (37°C) microscope slides, covered with a pre-warmed (37°C) coverslip and analysed by the CASA system, which consisted of an optical phase-contrast microscope (CX41; Olympus, Tokyo, Japan) with a warm stage (37°C) and a digital camera (avA1000–100 gc; Basler, Ahrensburg, Germany). Images were captured and analysed using SpermVision software (Minitube, Tiefenbach, Germany). Analysis was carried out using a 10× negative phase-contrast objective (PLN10XPH; Olympus). Data on total and progressive sperm motility was acquired, along with the following sperm kinetic values: average path velocity (VAP, μm s^{-1}), curvilinear velocity (VCL, μm s^{-1}), straight-line velocity (VSL, μm s^{-1}), lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz) and linearity (LIN). Software acquisition settings were set at a rate of 60 frames s^{-1} (60 Hz) with 30 frames acquired per field. A total of eight randomly-selected fields were acquired for each sample. Spermatozoa were considered immotile when the average orientation change (AOC) of the head was under 9.5°. Motility was considered to be stationary when AOC ≥ 9.5° and the distance in straight line (DSL) was under 6 μm. Motility was progressive when AOC ≥ 9.5° and DSL ≥ 6 μm.

Sperm viability was assessed by staining 10 μL of suspended spermatozoa with eosin–nigrosin (eosin Y yellow CI 45380, nigrosin CI 50420 dissolved in 0.9% NaCl; VWR International, Darmstadt, Germany) at a 1 : 1 ratio, incubating at room temperature for 2 min and then preparing smears that were air-dried before evaluation. At least 200 spermatozoa of each sample were evaluated with a light microscope (oil immersion; 1000×). White (unstained) spermatozoa were classified as live and those that showed pink or red coloration were classified as dead. Viability was also estimated using an image cytometer (see below).

Sperm aliquots were also fixed in Hancock's fixative and assessed for acrosome integrity and sperm morphology as previously described (Saragusty *et al.* 2009, 2010). At least 200 spermatozoa were evaluated by phase-contrast microscopy (oil immersion; 1000×). The acrosome was first evaluated according to the following categories: normal (intact acrosome), swollen, detaching and detached. Two hundred spermatozoa with intact acrosomes were then assessed for morphology and were classified as normal or as having a defect in the head, neck, midpiece or endpiece. Sperm morphology included searching for a wide range of abnormalities as previously described (Saragusty *et al.* 2010).

In parallel to the above, viability and concentration were assessed using an image cytometer (NucleoCounter SP-100; ChemoMetec, Allerød, Denmark). For concentration, an aliquot of sperm sample was diluted with Reagent S100 (ChemoMetec) at a known ratio and then loaded into SP1-Cassette (ChemoMetec). The reagent makes all cells permeable to the propidium iodide (PI) in the cassette. The cassette is then placed in the NucleoCounter SP-100 for counting. The NucleoCounter calculates concentration based on the dilution factor. For viability the same procedure is performed substituting phosphate-buffered saline (PBS) for Reagent S100. Only cells with a damaged membrane are stained with PI and the NucleoCounter calculates their fraction from the total cell number calculated when evaluating concentration.

Statistical analysis

Statistical analysis was performed using R (R Core Team 2014). Differences between the gradient centrifugation treatments in motility, viability, concentration, acrosome integrity, normal sperm morphology and yield of motile spermatozoa (motility × concentration × volume) from 1 mL of post-thaw sample, measured directly after centrifugation, were evaluated using the implementation of the Friedman test provided by the package Coin (Hothorn *et al.* 2008). This test accounts for the dependence of measurements derived from the sperm sample. For these analyses, the established 'manual' techniques for motility, concentration and viability evaluation were used. As we were dealing with a relatively small sample size (10 sperm samples), *P* values were computed based on approximation of the exact distribution of test statistics under the null hypothesis using 10 000 Monte-Carlo replications. Contrary to asymptotic tests that assume large sample sizes, this robust methodology does not require the computation of degrees of freedom to estimate *P* values. When the null hypothesis was rejected (i.e. $P < 0.05$), post-hoc pair-wise comparisons were performed using the implementation of the Wilcoxon signed-rank test provided by the package Coin. *P* values were computed using an exact methodology, which explains why degrees of freedom are not reported. *P* values were adjusted for multiple comparisons following the conservative Bonferroni correction. To evaluate the effect of treatments on sperm motility and kinetic variables, principal component analysis was performed on all nine motility descriptors.

A comparison of time points for motility evaluation was performed using Brunner's nonparametric test for longitudinal data using the package nparLD (Brunner *et al.* 2002; Noguchi *et al.* 2012). Following the authors' terminology, our experiment setting corresponds to a F0-LD-F2 design. This means that for sperm samples that were considered as subjects, there were no between-subject covariates and two within-subject covariates: time and treatment.

For comparison between the two evaluation methods ('manual' vs image cytometer or CASA) for concentration, viability and motility, Spearman correlation tests were used after combining all data from measurements performed directly after thawing (all treatments, all sperm samples).

Values are reported as mean ± s.d. and differences were considered to be significant when $P < 0.05$.

Results

Post-thaw evaluation revealed overall poor-quality samples with: motility 5.20 ± 2.71% (range 2–10%), viability 48.40 ± 11.86% (range 25–67%), concentration 154.99 ± 56.83 × 10^6 spermatozoa mL^{-1} (range 46.75 × 10^6–254.62 × 10^6), intact acrosome 58.40 ± 13.26% (range 26.0–71.5%) and normal morphology 78.90 ± 8.53% (range 69–95%).

Significant differences were detected between gradient centrifugation treatments for concentration, motility, sperm kinetic variables and viability (Table 1, Fig. 1), but not for the rate of acrosome integrity, normal sperm morphology or yield of motile spermatozoa (Table 2). While OptiPrep centrifugation treatment showed no improvement in motility over the Control, all other

Table 1. Sperm kinetic variables measured by computer-aided sperm analysis (CASA) system

Mean ± s.d. of sperm kinetic variables measured immediately after thawing and on resuspended sperm samples after separation by centrifugation through different density-gradient media. Statistical comparisons between pairs were performed using the exact Wilcoxon signed-rank test. Different superscript letters in the same row indicate statistically significant differences ($P < 0.05$). When strict corrections of all P values for multiple testing were done using the Bonferroni procedure no differences were detected. VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; ALH, lateral head displacement; BCF, beat cross frequency; LIN, linearity

Sperm kinetic variable	Control	Sham	Percoll	OptiPrep	Isolate	PureSperm
VAP (μm s^{-1})	32.19 ± 10.58^{d}	34.18 ± 6.04^{bcd}	44.28 ± 11.41^{ab}	31.40 ± 16.39^{abcd}	44.00 ± 7.13^{a}	36.89 ± 10.07^{cd}
VCL (μm s^{-1})	48.31 ± 18.46^{c}	59.28 ± 12.16^{b}	76.32 ± 19.94^{a}	55.10 ± 25.97^{abc}	71.52 ± 12.67^{a}	64.08 ± 18.54^{ab}
VSL (μm s^{-1})	27.27 ± 8.83^{c}	27.72 ± 5.17^{bc}	36.59 ± 10.36^{ab}	25.06 ± 13.85^{c}	37.34 ± 7.02^{a}	29.74 ± 8.43^{c}
ALH (μm)	2.78 ± 0.88^{a}	3.01 ± 0.58^{ab}	2.69 ± 0.54^{ab}	2.28 ± 1.08^{b}	3.02 ± 0.57^{ab}	2.81 ± 0.84^{ab}
BCF (Hz)	16.02 ± 6.46^{c}	18.32 ± 4.58^{c}	23.20 ± 5.22^{a}	16.41 ± 7.82^{bc}	21.88 ± 5.64^{ab}	19.61 ± 3.61^{bc}
LIN	0.58 ± 0.09^{a}	0.47 ± 0.09^{bc}	0.48 ± 0.07^{bc}	0.43 ± 0.09^{bc}	0.53 ± 0.08^{b}	0.47 ± 0.06^{c}

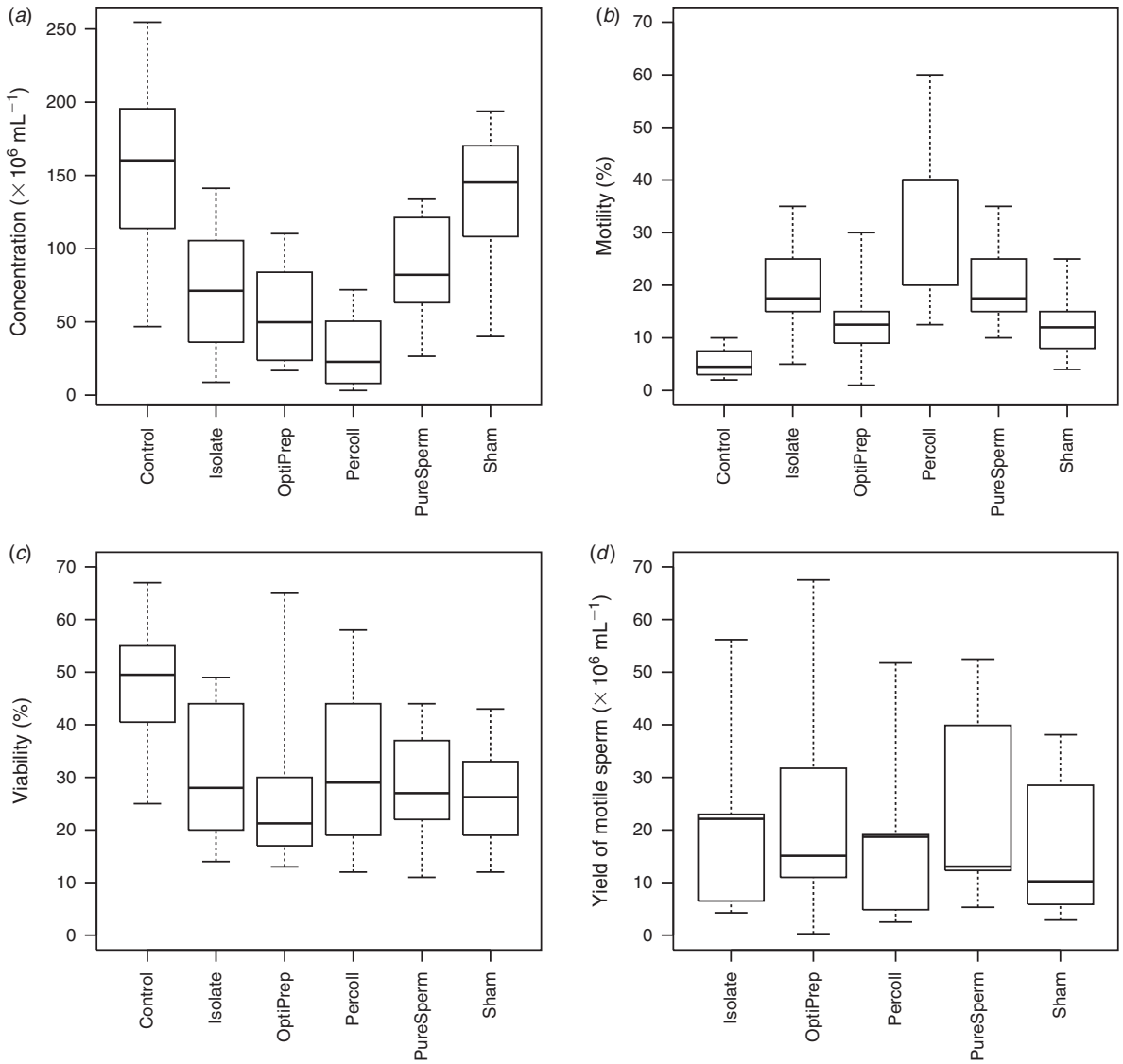

Fig. 1. Boxplot comparison showing the effect of different density-gradient centrifugation methods. In this figure, treatment effects on (*a*) concentration, (*b*) subjective motility, (*c*) viability by eosin–nigrosin staining of spermatozoa and (*d*) yield of (subjective) motile spermatozoa are shown. Boxes delimit the second and third quartiles (50% of central observations); the horizontal bar within each box represents the median; whiskers represent the minimal and maximal values. Yield values delineate the yield per 1 mL of post-thaw samples.

treatments significantly enhanced motility (Wilcoxon signed-rank tests: between Control and OptiPrep $P = 0.13$, between Control and all other methods $P < 0.02$). Subjective motility of the Percoll group was superior to all other treatments including the Control (Fig. 1*b*; Wilcoxon signed-rank tests: $P < 0.04$ for all) but not to PureSperm ($P = 0.059$). Motility was ≥30% (the minimum required for artificial insemination) in 6/10 of the Percoll samples, 2/10 of the PureSperm samples, 1/10 of both the Isolate and the OptiPrep samples and in none of the Sham or Control group samples. When motility was evaluated by CASA, differences between treatments were also detected (Table 2). The Percoll group was again the treatment for which average motility was the highest. However, differences were less pronounced with the CASA method and significance was only reached when Percoll was compared with the Control (Wilcoxon signed-rank tests: between Percoll and Control $P = 0.049$, between Control and all other methods $P > 0.069$). The CASA-derived motility characteristics for the different treatments are presented in Table 1 and Fig. 2. Results suggest that both the Percoll and the Isolate treatment groups exhibited high values in the various motility and kinetic variables, with PureSperm being intermediate while the Control, Sham and OptiPrep groups were generally low on all variables.

All centrifugation treatments significantly decreased concentration (Fig. 1*a*; Wilcoxon signed-rank tests with Bonferroni adjustment: between Control and Sham $P = 0.049$, between Control and all other methods $P < 0.01$). Viability of spermatozoa from all treatments was similar to that of the Control when

Table 2. Comparison of all density-gradient centrifugation treatments including Control

Differences between treatments were tested within sperm samples using the Friedman test. The number of data points considered for each analysis can be derived by multiplying the number of sperm samples by 6. CASA, computer-aided sperm analysis

Parameter	χ^2	*P*	*n* sperm samples
Concentration	43.1	<0.001	10
Subjective motility	33.5	<0.001	10
CASA total motility	20.0	<0.001	10
Motility after 3 h	30.8	<0.001	9
Viability by eosin–nigrosin	16.5	0.0031	10
Viability by NucleoCounter	16.5	0.0025	8
Acrosome integrity	5.43	0.37	10
Normal sperm morphology	2.99	0.72	9
Yield of motile spermatozoa (subjective)	3.52	0.52	5
Yield of motile spermatozoa (CASA)	5.60	0.25	5

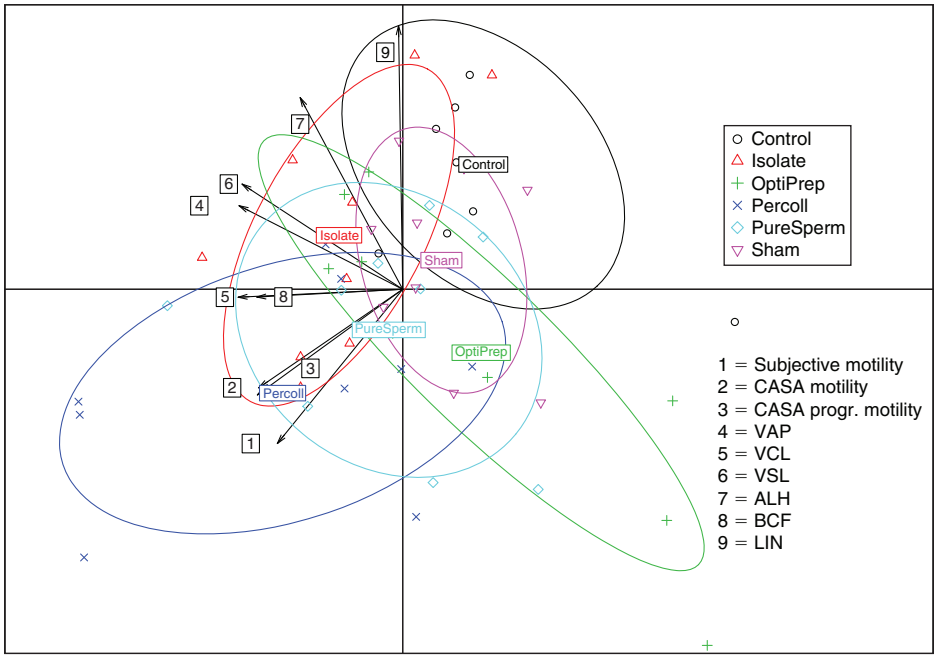

Fig. 2. Principal component analysis (PCA) plot. Plot showing the position of the different treatments in the space defined by the first two principal components of a principal component analysis performed on all nine motility parameters. These first two principal components capture more than 78% of the total variance (62.42% for *x*-axis, 15.95 for *y*-axis) is sufficient to justify expressing the original multidimensional data matrix in a two-dimensional representation. Arrows indicate vectors representing the direction of an increase in each individual sperm motility or kinetic descriptor. The longer the arrow is, the larger is the variance of the motility parameter. For each method, the oval contains ~2/3 of the data-points, representing the 1.5 inertia ellipse of the multivariate analysis. CASA, computer-aided sperm analysis; progr., progressive; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; ALH, lateral head displacement; BCF, beat cross frequency; LIN, linearity.

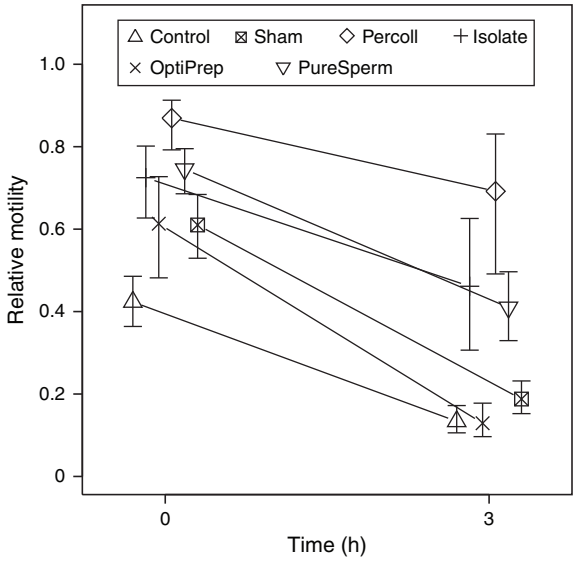

Fig. 3. Change in post-thaw subjective motility over time for the different density-gradient centrifugation methods. The *y*-axis is the conventional graphical representation of the nonparametric method used (see Methods). It represents the relative marginal effect of the different treatments across time, i.e. the probability that the value being considered presents higher motility than a random observation. The higher the value on the *y*-axis, the higher is the corresponding value of motility and the more effective is the treatment. By presenting relative, rather than absolute, motility values, the effect of the treatment through time can be inferred and not be overshadowed by between-sample differences. Intervals represent 95% confidence intervals of the relative marginal effects and can here be used to compare treatments, as the sample size is similar for each point.

it was evaluated by image cytometry (Wilcoxon signed-rank tests: between OptiPrep and Control $P = 0.12$, between Control and all other methods $P > 0.85$). When viability was evaluated by eosin–nigrosin staining there was no difference between centrifugation treatments (Friedman test without Control: $\chi^2 = 2.36$, $P = 0.69$) and all treatments were inferior to the Control group (Fig. 1*c*; Wilcoxon signed-rank tests: $P < 0.049$ for all). There were also no differences between treatments when yield of motile spermatozoa was compared based on CASA (Friedman test excluding Control: $\chi^2 = 5.60$, $P = 0.25$) or subjective (Friedman test excluding Control: $\chi^2 = 3.52$, $P = 0.52$) motility (Fig. 1*d*).

All samples were incubated at 37°C for 3 h after thawing. Overall, the different treatments influenced the extent to which motility decreased with time (Brunner test: ANOVA-type statistic (ATS) = 3.56, d.f. = 2.84, $P = 0.015$). Regardless of the time (0 h or 3 h), all methods except centrifugation with OptiPrep showed better motility than the Control (Fig. 3). Differences between treatments were noted at time 0 h (after centrifugation) as indicated above. Percoll remained the best method after 3 h along with Isolate (Wilcoxon signed-rank tests: between Percoll and Isolate $P = 0.35$, between Percoll and all other methods $P < 0.04$). Percoll was also the method for which the decrease in motility over time was the smallest (Fig. 3).

Comparison between the established 'manual' evaluation techniques and the automated systems found the highest correlation between concentration estimated by haemocytometer counting and the NucleoCounter (Fig. 4*a*; Spearman correlation: rho = 0.92, $P < 0.001$). For viability the correlation was still significant but not as high (Fig. 4*b*; Spearman correlation: rho = 0.57, $P < 0.001$). Correlation was also good when subjective motility was correlated with either total or progressive motility evaluated by CASA (Fig. 4*c*; total motility: rho = 0.66, $P < 0.001$; progressive motility: rho = 0.72, $P < 0.001$).

Discussion

The idea of linking wild and captive populations by tapping the wild for genetic material that can be used to increase the genetic diversity of captive (or isolated) populations is not new but rarely practiced (Howard *et al.* 2002; Hildebrandt *et al.* 2009, 2012). Transporting semen samples is undoubtedly the easiest and least invasive method for moving genetic material between populations and it is relatively simple to collect by electroejaculation. Samples collected, however, will have to be cryopreserved at or near the place of collection since the distance to the location and time of use does not normally permit chilled storage. Furthermore, as we have experienced with respect to semen collected from wild African savanna elephant (*Loxodonta africana*) bulls (Hermes *et al.* 2013), movement of such biological material between countries and continents is bound by much red tape and requires extensive time-consuming testing before importation. When it comes to rare or endangered species, established cryopreservation protocols are often not in place and experiments are required before a customised protocol is devised. In the process of experimenting, not all samples survive well. However, discarding these would be a huge waste of important material. We were faced with this dilemma with respect to the African elephant semen we had collected and cryopreserved (Hermes *et al.* 2013). Before finding the right protocol we had amassed ~1700 mL of extended and frozen semen that was of a quality not suitable for use in artificial insemination programs. This motivated us to search for ways to improve the quality of what we had on our hands and density-gradient centrifugation was one possible option. However, which of the many gradients available in the market would be the most suitable for the compromised frozen–thawed elephant spermatozoa on our hands was not known. We therefore selected the gradients either because of their reported success rate in other species (Claassens *et al.* 1998; Mousset-Siméon *et al.* 2004) or because they represent different families (Isolate and PureSperm are made of colloidal saline-coated silica particles, Percoll is made of colloidal polyvinylpyrrolidon (PVP)-coated silica particles while OptiPrep is made of non-ionic iodixanol).

Percoll gradient was long reported to primarily select for motile spermatozoa with better functionality (McClure *et al.* 1989). This was evident in our study as well. In comparison to all other treatments, Percoll resulted in the highest motility. This higher motility remained relatively unchanged over the time of incubation suggesting that the quality of the spermatozoa selected was superior to the other techniques. The ability of the spermatozoa to remain motile for such a long time at 37°C suggests that such cells may have the stamina to travel through the female reproductive tract to the site of fertilisation.

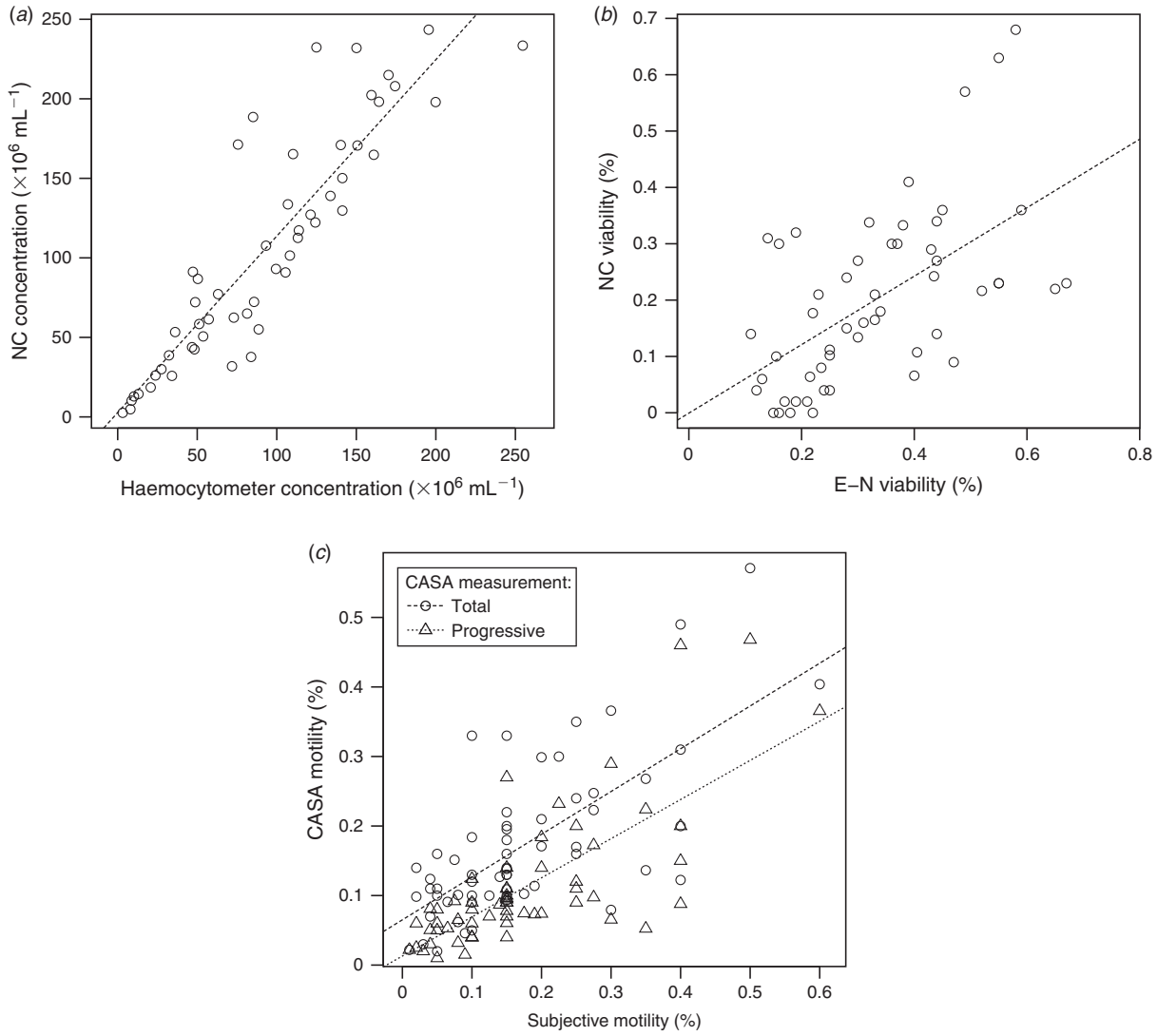

Fig. 4. Comparison between different evaluation methods. Plotted are comparisons between (*a*) NucleoCounter (NC) and haemocytometer for concentration evaluation, (*b*) NC and eosin–nigrosin (E–N) staining for viability evaluation and (*c*) computer-aided sperm analysis (CASA) and subjective evaluation for sperm motility. One outlier (6.05 s.d. above the overall mean), likely resulting from a measurement error, was not plotted on plots (*a*) and (*b*) for graphical purposes. It was considered in the analysis; however, its presence or absence does not significantly influence our results and conclusions. Regression lines are displayed for graphical purposes only.

Also similar to the finding in previous studies (Claassens *et al.* 1998; Mousset-Siméon *et al.* 2004), the most noticeable improvement was in sperm motility and of all tested media, OptiPrep results were the least favourable. To varying degrees, all these selection techniques come with a price – loss of a large proportion of the cells in the sample. Naturally, some of these cells should be removed by the density gradient because they are dead or otherwise compromised. However, some were lost because they remained in the medium and were aspirated with it. At the centrifugation force used in this study, the consistency of the pellet differed between media, with that in Percoll being the most diffused one. For these reasons, while all treatments showed a vast loss of cells, this was most notable in the Percoll treatment. The density of the gradient cannot explain this outcome as, in comparison to Percoll, both Isolate and PureSperm have lower density while OptiPrep has higher density.

In 1996 Percoll was withdrawn from the market for human clinical use, possibly because of reports on the presence of endotoxins at levels beyond the acceptable limits in some batches of the medium (Svalander *et al.* 1995). Furthermore, endotoxins were reported to have a negative effect on the implantation, development and DNA integrity of human embryos (Nagata and Shirakawa 1996; Jaiswal *et al.* 2009). Such effects, however, were not found when Percoll was evaluated directly. In a study on mouse embryos and human spermatozoa, Scott and Smith (1997) did not find any negative effects. It is possible that, as in many other instances, there are differences in sensitivity between species and between cell types. It is also possible that

the batch they had used did not contain endotoxins at damaging levels. In our study Percoll seemed to have no negative effect on elephant sperm motility or viability. Samples selected by Percoll gradient showed almost no decline in motility over 3 h of incubation. Whether elephant embryos are sensitive to endotoxins and to what extent is, at present, impossible to evaluate since to date *in vitro* fertilisation and embryo culture are not reported in this species. What the effects of Percoll on the elephant uterus and embryo implantation still needs to be investigated. During artificial insemination in elephants the semen is usually deposited into the vagina near the cervical opening. We can thus speculate that the small residues of Percoll and the very small residues of endotoxins therein, if such are actually present, would probably be filtered out during the passage of spermatozoa through the cervix, the uterine body and the uterine horns. With the length and size of the reproductive system in elephants, such small residues would be vastly diluted out to the point of (probably) having no negative effects.

At present we do not have an explanation as to why viability decreased in all treatments by 15–20% compared with the Control. If it were to do with the centrifugation media then we would have expected the sham centrifugation, for which no media was added, to show viability similar to that of the Control group. The only difference between them is that the freezing medium with the glycerol in the Control group was replaced by the same medium without glycerol in the Sham group. The other factor that could account for this drop in viability is the centrifugation procedure. Exposing spermatozoa to centrifugation force of only 400*g*, however, should not inflict such damage on the cells. It could, however, be that the poor outcome of the freezing procedure may have caused, amongst other things, higher fragility of the cells so that even a mild centrifugation force was enough to kill them. This aspect of the process will have to be further investigated.

One of the additional goals of our study was to evaluate the use of CASA and an image cytometer to estimate elephant sperm motility, concentration and viability. Although correlation was found between these techniques and the established manual techniques used in our laboratory, only the correlation for concentration was very strong. With the CASA system used (SpermVision), many non-cell particles were identified as stationary motile or immotile spermatozoa and had to be removed manually one-by-one. This considerably reduces the accuracy of the system. Furthermore, because this process took time and could not be done after the data had been saved, motility of the other samples awaiting analysis dropped, making it even less accurate and thus resulting in the lower values often observed. Still, spermatozoa must be motile to reach the fertilisation site and succeed in fertilising the oocytes. Several studies attempted to associate various sperm motility and kinetic variables derived from the CASA analysis with the fertilising capacity of those samples. Different studies, even when conducted on the same species, resulted in contrasting results (Holt *et al.* 1997; Broekhuijse *et al.* 2012). With the exception of ALH, for which the treatments in the present study showed high similarity, the Percoll and Isolate treatments tended to have higher values in the various motility and kinetic parameters. Increase in VCL and decrease in LIN have been suggested to be the most accurate predictors of hyperactivated motility (Cancel *et al.* 2000). It was also previously suggested that Percoll separation selects for hyperactivated spermatozoa with improved longevity (Moohan and Lindsay 1995). In this study samples selected by Percoll exhibited kinetic characteristics associated with hyperactivation, and Percoll also proved to select for spermatozoa that maintained motility over 3 h of incubation at 37°C.

The reason concentration estimated by the nuclear counter was highly correlated with that estimated by the counting chamber but viability had much lower correlation to the established eosin–nigrosin staining technique is most likely due to the way the nuclear counter makes its calculations. To estimate concentration, an aliquot of the sample is suspended in a solution that makes all cells permeable. Cells are then stained with propidium iodide in the cassette into which they are loaded and then counted. This means that as long as the aliquot used was representative, the estimation will reflect the actual concentration. For viability, a second aliquot is taken from the original sample, suspended in PBS and then loaded into a second cassette where it is stained with propidium iodide and then counted. This second counting is then divided by the first counting to generate percentage of dead cells and viability is calculated from this. This counting process introduces at least three possible errors. The first is that the two aliquots may not be identical, thus resulting in an inaccurate value. The second is the fact that only dead cells are stained. Normally fluorescence viability kits contain a stain for dead cells and a stain for live ones. This is because the assumption that all cells that have not been stained by PI would have been stained by the other stain (e.g. SYBR-14) is not necessarily true. Third, spermatozoa of different species respond differently to PBS. It is thus possible that some of the cells lost viability as a result of their exposure to PBS. Our finding that the values produced by the image cytometer were consistently higher than those of the eosin–nigrosin staining, and the higher the value the larger the difference, provides further support to all these factors. This somewhat questionable viability assessment may explain why in a conference poster (Egeberg *et al.* 2013*a*) presented by a team of researchers that included employees from the company producing this image cytometer, viability was included and yet, in a paper published later by the same group (Egeberg *et al.* 2013*b*) viability was completely omitted. Viability assessment can be done by alternative methods such as the hypo-osmotic swelling test, eosin–nigrosin or fluorescence staining. Fluorescence staining can also be used for automated viability evaluation by any of several CASA systems or by flow cytometry.

Several different gradient centrifugation treatments and media were evaluated in a search for the optimal selection process for African savannah elephant spermatozoa. Of the options tested Percoll seemed to result in a better outcome due to its superior motility in a large proportion of the samples and the ability of the selected spermatozoa to maintain their motility over time. For the purpose of artificial insemination and the resultant fertilisation, such cells might have better chance to succeed. Because of the poor quality of the samples to start with and the low yield of these gradients, several samples will need to be thawed, treated and combined for each artificial insemination

procedure. With two offspring already on the ground and an ongoing pregnancy from semen collected in Africa from wild elephant bulls (T. B. Hildebrandt, R. Hermes, J. Saragusty, R. Potier and F. Göritz, unpubl. data; Hildebrandt *et al.* 2012), we are hopeful that such treatment will help save some of the spermatozoa in these poor-quality samples so that the genetic pool of the captive African savannah elephant can be further enriched.

Acknowledgements

The authors wish to express their gratitude to the following people and organisations who contributed to the wild elephant semen collection project: Paul Bartels, Barbara Baker, Dr Douw Grobler, J. J. Altana, The Phinda Private Game Reserve, Republic of South Africa and the staff from the Pittsburgh Zoo, especially Willie Theison. We would like to express our gratitude to the Association Beauval Conservation et Recherche (ABCR), adjunct to the Beauval Zoo, and to Robert and Kim Fanelli of Riverview Homes, Inc., USA for their generous financial support of the semen collection and cryopreservation project. We would also like to thank Mrs. Jette Ziep from the Leibniz Institute for Zoo and Wildlife Research for her technical assistance.

References

Blottner, S. (1998). Semen preservation for reproduction management in rare and endangered species. *Adv. Ethol.* **s33**, 9–13.

Bouché, P., Douglas-Hamilton, I., Wittemyer, G., Nianogo, A. J., Doucet, J.-L., Lejeune, P., and Vermeulen, C. (2011). Will elephants soon disappear from West African savannahs? *PLoS ONE* **6**, e20619. doi:10.1371/JOURNAL.PONE.0020619

Broekhuijse, M. L. W. J., Šoštarić, E., Feitsma, H., and Gadella, B. M. (2012). Application of computer-assisted semen analysis to explain variations in pig fertility. *J. Anim. Sci.* **90**, 779–789. doi:10.2527/JAS.2011-4311

Brunner, E., Domhof, S., and Langer, F. (2002). 'Nonparametric Analysis of Longitudinal Data in Factorial Experiments'. (John Wiley & Sons, Inc.: New York.)

Cancel, A. M., Lobdell, D., Mendola, P., and Perreault, S. D. (2000). Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. *Hum. Reprod.* **15**, 1322–1328. doi:10.1093/HUMREP/15.6.1322

Claassens, O. E., Menkveld, R., and Harrison, K. L. (1998). Evaluation of three substitutes for Percoll in sperm isolation by density-gradient centrifugation. *Hum. Reprod.* **13**, 3139–3143. doi:10.1093/HUMREP/13.11.3139

Colleoni, S., Lagutina, I., Lazzari, G., Rodriguez-Martinez, H., Galli, C., and Morrell, J. M. (2011). New methods for selecting stallion spermatozoa for assisted reproduction. *J. Equine Vet. Sci.* **31**, 536–541. doi:10.1016/J.JEVS.2011.03.009

Crosier, A. E., Pukazhenthi, B. S., Henghali, J. N., Howard, J., Dickman, A. J., Marker, L., and Wildt, D. E. (2006). Cryopreservation of spermatozoa from wild-born Namibian cheetahs (*Acinonyx jubatus*) and influence of glycerol on cryosurvival. *Cryobiology* **52**, 169–181. doi:10.1016/J.CRYOBIOL.2005.10.011

Egeberg, D. L., Kjærulff, S., Hansen, C., Petersen, J. H., Glensbjerg, M., Skakkebæk, N. E., Jørgensen, N., and Almstrup, K. (2013*a*). Assessment of sperm concentration and viability by automated image cytometry. *Andrology* **1**, 53. doi:10.1111/J.2047-2927.2012.00065.X

Egeberg, D. L., Kjærulff, S., Hansen, C., Petersen, J. H., Glensbjerg, M., Skakkebæk, N. E., Jørgensen, N., and Almstrup, K. (2013*b*). Image cytometer method for automated assessment of human sperm concentration. *Andrology* **1**, 615–623. doi:10.1111/J.2047-2927.2013.00082.X

Hermes, R., Saragusty, J., Göritz, F., Bartels, P., Potier, R., Baker, B., Streich, W. J., and Hildebrandt, T. B. (2013). Freezing African elephant semen as a new population management tool. *PLoS ONE* **8**, e57616. doi:10.1371/JOURNAL.PONE.0057616

Hildebrandt, T. B., Roellig, K., Goeritz, F., Fassbender, M., Krieg, R., Blottner, S., Behr, B., and Hermes, R. (2009). Artificial insemination of captive European brown hares (*Lepus europaeus* PALLAS, 1778) with fresh and cryopreserved semen derived from free-ranging males. *Theriogenology* **72**, 1065–1072. doi:10.1016/J.THERIOGENOLOGY.2009.06.026

Hildebrandt, T. B., Hermes, R., Saragusty, J., Potier, R., Schwammer, H. M., Balfanz, F., Vielgrader, H. D., Baker, B., Bartels, P., and Göritz, F. (2012). Enriching the captive elephant population genetic pool through artificial insemination with frozen–thawed semen collected in the wild. *Theriogenology* **78**, 1398–1404. doi:10.1016/J.THERIOGENOLOGY.2012.06.014

Holt, C., Holt, W. V., Moore, H. D. M., Reed, H. C. B., and Curnock, R. M. (1997). Objectively-measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. *J. Androl.* **18**, 312–323. doi:10.1002/J.1939-4640.1997.TB01925.X

Hothorn, T., Hornik, K., van de Wiel, M. A., and Zeileis, A. (2008). Implementing a class of permutation tests: the coin package. *J. Stat. Softw.* **28**, 1–23.

Howard, J. G., Marker, L., Pukazhenthi, B. S., Roth, T. L., Swanson, W. F., Grisham, J., and Wildt, D. E. (2002). Genome resource banking and successful artificial insemination with cryopreserved spermatozoa in the cheetah. In 'Proceedings of the 9th International Symposium on Spermatology, Cape Town'. (Eds G. Van Der Horst, D. Franken, R. Bornman, T. De Jager and S. Dyer.) p. 70. (Monduzzi Editore International Proceedings Division: Bologna.)

Jaiswal, Y. K., Jaiswal, M. K., Agrawal, V., and Chaturvedi, M. M. (2009). Bacterial endotoxin (LPS)-induced DNA damage in preimplanting embryonic and uterine cells inhibits implantation. *Fertil. Steril.* **91**, 2095–2103. doi:10.1016/J.FERTNSTERT.2008.04.050

Kiso, W. K., Asano, A., Travis, A. J., Schmitt, D. L., Brown, J. L., and Pukazhenthi, B. S. (2012). Pre-treatment of Asian elephant (*Elephas maximus*) spermatozoa with cholesterol-loaded cyclodextrins and glycerol addition at 4°C improves cryosurvival. *Reprod. Fertil. Dev.* **24**, 1134–1142. doi:10.1071/RD11266

Lei, R., Brenneman, R. A., and Louis, E. E. (2008). Genetic diversity in the North American captive African elephant collection. *J. Zool. (Lond.)* **275**, 252–267. doi:10.1111/J.1469-7998.2008.00437.X

Leimgruber, P., Senior, B., Uga, Aung, M., Songer, M. A., Mueller, T., Wemmer, C., and Ballou, J. D. (2008). Modelling population viability of captive elephants in Myanmar (Burma): implications for wild populations. *Anim. Conserv.* **11**, 198–205. doi:10.1111/J.1469-1795.2008.00172.X

McClure, R. D., Nunes, L., and Tom, R. (1989). Semen manipulation: improved sperm recovery and function with a two-layer Percoll gradient. *Fertil. Steril.* **51**, 874–877.

Montano, G. A., Kraemer, D. C., Love, C. C., Robeck, T. R., and O'Brien, J. K. (2011). *In vitro* function of frozen–thawed bottlenose dolphin (*Tursiops truncatus*) spermatozoa undergoing sorting and recryopreservation. *Reprod. Fertil. Dev.* **23**, 240. [Abstract] doi:10.1071/RDV23N1AB284

Moohan, J. M., and Lindsay, K. S. (1995). Spermatozoa selected by a discontinuous Percoll density gradient exhibit better motion characteristics, more hyperactivation and longer survival than direct swim-up. *Fertil. Steril.* **64**, 160–165.

Mousset-Siméon, N., Rives, N., Masse, L., Chevallier, F., and Mace, B. (2004). Comparison of six density-gradient media for selection of cryopreserved donor spermatozoa. *J. Androl.* **25**, 881–884. doi:10.1002/J.1939-4640.2004.TB03157.X

Nagata, Y., and Shirakawa, K. (1996). Setting standards for the levels of endotoxin in the embryo culture media of human *in vitro* fertilisation and embryo transfer. *Fertil. Steril.* **65**, 614–619.

Nicolas, M., Alvarez, M., Borragán, S., Martinez-Pastor, F., Chamorro, C. A., Alvarez-Rodriguez, M., de Paz, P., and Anel, L. (2012). Evaluation of the qualitative and quantitative effectiveness of three media of centrifugation (Maxifreeze, Cushion Fluid Equine and PureSperm 100) in preparation of fresh or frozen–thawed brown bear spermatozoa. *Theriogenology* **77**, 1119–1128. doi:10.1016/J.THERIOGENOLOGY.2011.10.016

Noguchi, K., Gel, Y. R., Brunner, E., and Konietschke, F. (2012). nparLD: an R software package for the nonparametric analysis of longitudinal data in factorial experiments. *J. Stat. Softw.* **50**, 1–23.

Pukazhenthi, B., Laroe, D., Crosier, A., Bush, L. M., Spindler, R., Pelican, K. M., Bush, M., Howard, J. G., and Wildt, D. E. (2006). Challenges in cryopreservation of clouded leopard (*Neofelis nebulosa*) spermatozoa. *Theriogenology* **66**, 1790–1796. doi:10.1016/J.THERIOGENOLOGY.2006.02.020

R Core Team (2014). R: a language and environment for statistical computing. (R Foundation for Statistical Computing: Vienna, Austria). Available at http://www.R-project.org/ [Verified 18 February 2015].

Saragusty, J. (2012). Elephants under human care: fertility, birth rate, infant mortality and survivorship. In 'Elephants: Ecology, Behaviour and Conservation'. (Eds M. Aranovich and O. Dufresne.) pp. 1–47. (Nova Publishers: New York.)

Saragusty, J., Hildebrandt, T. B., Behr, B., Knieriem, A., Kruse, J., and Hermes, R. (2009). Successful cryopreservation of Asian elephant (*Elephas maximus*) spermatozoa. *Anim. Reprod. Sci.* **115**, 255–266. doi:10.1016/J.ANIREPROSCI.2008.11.010

Saragusty, J., Walzer, C., Petit, T., Stalder, G., Horowitz, I., and Hermes, R. (2010). Cooling and freezing of epididymal spermatozoa in the common hippopotamus (*Hippopotamus amphibius*). *Theriogenology* **74**, 1256–1263. doi:10.1016/J.THERIOGENOLOGY.2010.05.031

Scott, L., and Smith, S. (1997). Mouse *in vitro* fertilisation, embryo development and viability and human sperm motility in substances used for human sperm preparation for assisted reproduction. *Fertil. Steril.* **67**, 372–381. doi:10.1016/S0015-0282(97)81925-5

Svalander, R., Lundin, K., and Holmes, P. V. (1995). Endotoxin levels in Percoll density-gradient media used to prepare spermatozoa for human IVF treatment. *Hum. Reprod.* **10**, 130. [Abstract]

Van der Zwalmen, P., Bertin-Segal, G., Geerts, L., Debauche, C., and Schoysman, R. (1991). Sperm morphology and IVF pregnancy rate: comparison between Percoll-gradient centrifugation and swim-up procedures. *Hum. Reprod.* **6**, 581–588.