Asian elephant (*Elephas maximus*) seminal plasma: establishing the proteome and effect on spermatozoa when added to cryomedium

Cameron Negus[A],*[ID], Anuchai Pinyopummin[B][ID], Sittidet Mahasawangkul[C][ID], Rebecca Hobbs[D][ID] and Roslyn Bathgate[A][ID]

For full list of author affiliations and declarations see end of paper

*Correspondence to:
Cameron Negus
Faculty of Science, Sydney School of Veterinary Science, The University of Sydney, Sydney, NSW, Australia
Email: cameron.negus@sydney.edu.au

Handling Editor:
Marc Yeste

ABSTRACT

Context. The removal or supplementation of ejaculates with seminal plasma (SP) can affect cryotolerance and post-thaw survival of spermatozoa in many species. In the Asian elephant (*Elephas maximus*), elucidation of the SP proteome and investigation of how it affects spermatozoa may enable improvement of cryopreservation protocols. **Aims.** Herein, we characterise the Asian elephant SP proteome and investigate the impacts of SP on sperm cryotolerance in the presence of conspecific or heterospecific SP. **Methods.** Proteomic analysis of Asian elephant SP was performed using mass spectrometry on nine samples from three individuals. In a separate study, SP was removed from six ejaculates and spermatozoa were resuspended in Tris extender supplemented with: no seminal plasma (NOSP), conspecific SP from ejaculates exhibiting 'good' (GSP, >60%) or mixed sperm total motility (MSP), or horse SP (HSP). Samples underwent cryopreservation, and sperm parameters were compared prior to cryopreservation and after thawing (0 and 2 h). **Key results.** Mass spectrometry identified 155 proteins from an array of families. Significant differences were observed in post-thaw sperm quality between SP treatments: high concentrations of MSP (25%, v/v) displayed greater average path and straight-line velocity immediately after thawing ($P < 0.05$) and greater sperm motility index and beat cross frequency than NOSP after 2 h post-thaw incubation ($P < 0.05$). The addition of HSP improved sperm kinematic parameters compared to NOSP and GSP treatments ($P < 0.05$). **Conclusions and implications.** These preliminary findings suggest the potential of SP to enhance the cryosurvival of Asian elephant spermatozoa, with HSP showing particularly promising results compared to conspecific SP (GSP). Further research into the specific effects of Asian elephant SP proteins is warranted.

Keywords: Asian elephant, cryopreservation, *Elephas maximus*, mass spectrometry, preservation, proteins, semen, seminal plasma.

Introduction

The Asian elephant (*Elephas maximus*) is categorised by the IUCN (Williams *et al.* 2020) as endangered with declining numbers, and captive breeding is a vital component of preserving this species. The management of captive Asian elephants incorporates assisted reproductive techniques such as artificial insemination (AI) with fresh and chilled preserved semen to supplement natural breeding. This is in an effort to improve reproductive output, enable outbreeding to enhance genetic diversity within isolated populations, and thus ensure the sustainability of captive populations (Hildebrandt *et al.* 2006). However, the use of cryopreserved Asian elephant semen has been unsuccessful, attributed to inconsistent quality of collected semen (Hildebrandt *et al.* 2000) and poor post-thaw sperm survival. Despite multiple attempts using semen samples displaying ≥40% post-thaw motility, no live Asian elephant calves have been born from frozen-thawed artificially inseminated semen (Thongtip *et al.* 2009). Although research suggests methods for cryo-sensitive spermatozoa (O'Brien *et al.* 2013), cryopreservation of Asian elephant semen

Collection: Dedication to Jim Cummins

still requires fundamental improvements in processing and freezing techniques to achieve success after AI.

An ejaculate is composed of spermatozoa and the acellular seminal plasma (SP), primarily secreted from the accessory sex glands (Flint *et al.* 2015). Seminal plasma is a complex medium of inorganic ions, sugars, organic salts, enzymes, proteins and various other factors. It is involved in a multitude of sperm functions and processes preceding fertilisation, as reviewed by Juyena and Stelletta (2012), though the constituents are highly species-dependent. Within the elephant species, the seminal vesicles are the largest accessory sex glands and believed to contribute the greatest proportion of volume to the SP in the ejaculate, as indicated by a large reduction in internal fluid volume of the seminal vesicles observed via ultrasonography after ejaculation (Hildebrandt *et al.* 2000). Similarly, in stallions, seminal vesicles also significantly decrease in size after ejaculation (Weber *et al.* 1990). Although similar effects have been recorded in the stallion ampulla, these accessory sex glands are shown to vary in size between breeds and seasons (Pozor and McDonnell 2002).

The protein component of SP has recently become a focal point of reproductive research in domestic animals (Rickard *et al.* 2015; Perez-Patiño *et al.* 2016; Aquino-Cortez *et al.* 2017), as SP proteins are known to contribute to the fertilisation process by remodelling the sperm surface (Mogielnicka-Brzozowska and Kordan 2011; Rodríguez-Martínez *et al.* 2011). Several studies have demonstrated that specific SP proteins influence the function and fertilising capacity of spermatozoa (Moura *et al.* 2006; Vilagran *et al.* 2015) and, in some species, are utilised as markers of sperm freezability (Jobim *et al.* 2011; Vilagran *et al.* 2015). Minimal research has been done on the proteomics of Asian elephant SP. One- and two-dimensional gel electrophoresis have revealed a large protein component of Asian elephant SP collected via manual rectal massage technique, but gel spots varied greatly in size between ejaculates (Kiso *et al.* 2013). A recent non-targeted proteomic analysis using tandem-mass spectrometry revealed 1183 proteins in Asian elephant SP, of which 597 proteins were mapped to identified proteins from 58 species, with only a small proportion (29 proteins) recognised to be related to reproductive processes (Wattananit *et al.* 2023). Continuing to establish an Asian elephant SP proteome will likely make a significant contribution to the development of sperm cryo-protocols and guide selection of males and semen samples for AI and breeding programs.

The role and benefits of SP when applying sperm preservation techniques, such as cryopreservation, is still under debate, often with varying results observed across species. For example, horse spermatozoa are able to maintain greater motility during cooled storage and cryopreservation when SP is removed prior (Jasko *et al.* 1991; Moore *et al.* 2005). However, for other species, such as humans (Ben *et al.* 1997), goats (Azerêdo *et al.* 2001), and red deer (Martínez-Pastor *et al.* 2006), SP inclusion has proven to improve sperm cryosurvival. Even within species, the reported effects of SP

during cryopreservation are conflicting. While the beneficial effects of SP removal prior to freezing have already been shown with stallions and boars (Kawano *et al.* 2004; Moore *et al.* 2005), other studies in the same species have demonstrated that SP obtained from certain males (classified as 'good freezers'; ones demonstrating high post-thaw quality) may improve the cryosurvival of spermatozoa from other males (Aurich *et al.* 1996; Hernández *et al.* 2007). Similarly, the addition of SP from infertile bulls can reduce the fertilising ability of spermatozoa from bulls with high fertility (Henault and Killian 1996), which is hypothesised to be due to differences in protein composition of the SP (Zahn *et al.* 2005; Vilagran *et al.* 2015). Because of the high variability of sperm quality seen in fresh Asian elephant ejaculates (Hildebrandt *et al.* 2000), it is possible that the SP composition, including proteins, may be highly variable and strongly influence the observed sperm quality (Kiso *et al.* 2013).

Previous studies have advocated for removal of Asian elephant SP prior to cryopreservation (Saragusty *et al.* 2009). However, others have retained the SP during freezing with similar success (Thongtip *et al.* 2004, 2009). The beneficial effects of Asian elephant SP have been more clearly demonstrated during chilled liquid storage where sperm motility and acrosome integrity were better maintained in the presence of SP compared to its absence (Pinyopummin *et al.* 2017). Further studies directly comparing the effects of SP, and source of ejaculate quality, on Asian elephant spermatozoa during cryopreservation are required to understand the role of SP during and after the freezing process.

Studies on cryopreservation of spermatozoa using heterospecific SP have demonstrated beneficial *in vitro* effects. For example, rainbow trout (Ustuner *et al.* 2016) and dog (Mataveia *et al.* 2010) SP have been shown to improve ram spermatozoa cryosurvival. Horse SP (HSP) has previously been added to Asian elephant spermatozoa during chilled storage and has demonstrated greater preservation of sperm motility and velocity compared to conspecific SP (Pinyopummin *et al.* 2017). Furthermore, HSP was found to reverse the detrimental effects of high dilution rates on Asian elephant sperm motility during chilled storage (Pinyopummin *et al.* 2018). Despite this, the cryoprotective effects of HSP on Asian elephant spermatozoa have not previously been investigated.

The aims of this study were to undertake two preliminary investigations to improve the cryosurvival of Asian elephant spermatozoa by addition of SP proteins. Working within the constraints of limited samples from this species, the first investigation aimed to characterise the Asian elephant SP proteome using mass spectrometry. The second part aimed to investigate the effects of the presence and absence of both con- and heterospecific SP on Asian elephant spermatozoa during cryopreservation. This study serves as a first step towards more thorough investigation of the effect of specific proteins on Asian elephant spermatozoa during cryopreservation, contributing to the development of more effective assisted reproductive technologies for this endangered species.

Materials and methods

Experimental design

This study is presented as two experimental designs: (1) proteomic analysis of Asian elephant seminal plasma (Fig. 1a), and (2) cryopreservation of semen samples with and without con- and heterospecific SP (Fig. 1b).

Animals

All experimental procedures were carried out with the approval of the Animal Ethics Committee of the Taronga Conservation Society Australia (4a/04/114), the Animal Usage and Ethics Committee of Kasetsart University (ACKU 01858), and endorsed by the University of Sydney Animal Ethics Committee (2016/1010).

For the SP proteomic study, three sexually mature Asian elephant bulls (14–40 years of age) housed at different zoological institutions across Australia were used for semen collection. Animals were given water *ad libitum* and fed a varied diet as managed by each institution, including mostly hay and lucerne, along with treats of fruit, vegetables, bread, sugar cane, bamboo, and leafy branches. Elephants used in this study had proven fertility. Ejaculate samples were collected from these males between May 2013 and October 2015.

For the cryopreservation study, nine sexually mature Asian elephant bulls (20–35 years of age) housed at the National Elephant Institute of the Forest Industry Organization, Lampang, Thailand (latitude 18°21.60′N and longitude 99°14.92′E) were used for semen collection. The animals were fed a mixture of pangola grass, sugarcane, banana, and corn, and supplemented with concentrated feed (8% protein, 2% fat, and 20% fibre). Elephants used in this study varied in terms of proven fertility. Ejaculate samples were collected from these males and analysed between August and September 2018.

Semen collection and processing

Semen samples were collected by manual rectal massage method (Schmitt and Hildebrandt 1998). To avoid urine contamination, semen collecting tubes were changed frequently during the collection process, and samples were collected in multiple fractions. Fractions with urine contamination as determined by colour and odour were discarded. Non-contaminated fractions were pooled as a single sample for further processing. Semen volume and pH (pH indicator strips; Universal indicator, Merck, Germany), sperm concentration (Neubauer hemocytometer; Evans *et al.* 1987), and the percentage of spermatozoa with normal viability, functional plasma membranes, and normal acrosome integrity were determined with fresh semen
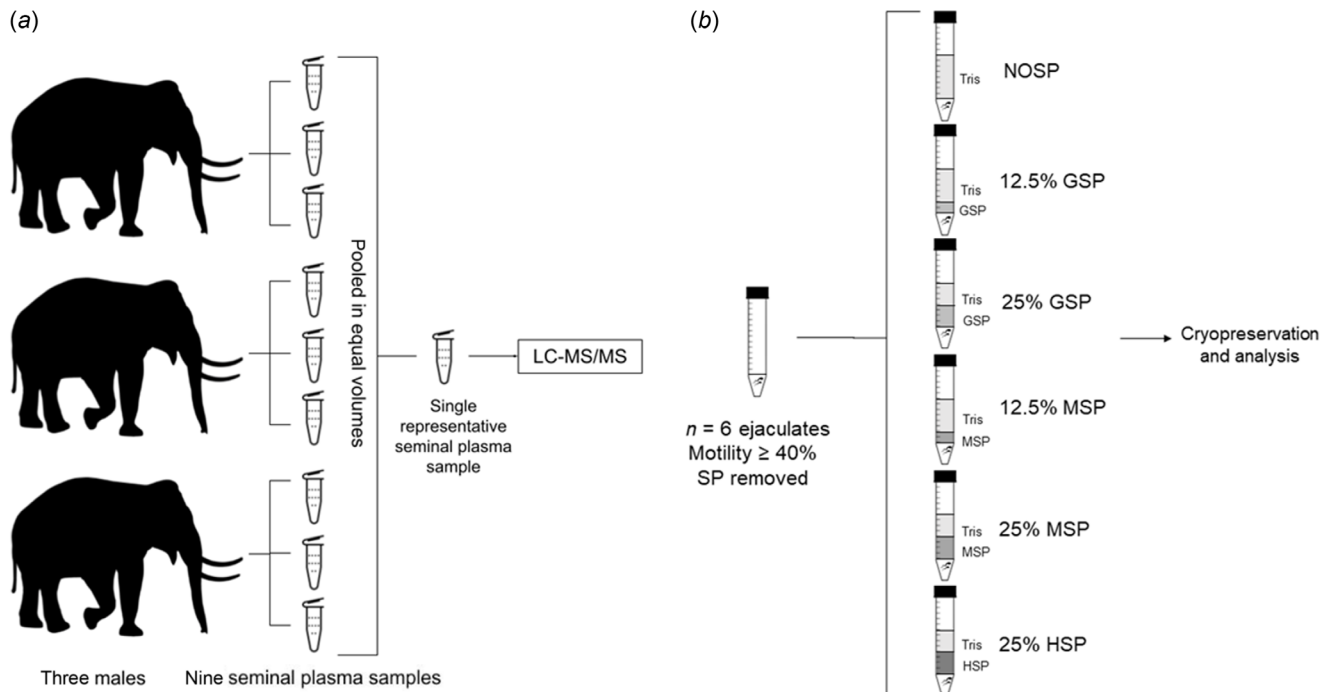

Fig. 1. Experimental designs for the study. (a) Proteomic analysis of Asian elephant seminal plasma. Seminal plasma samples were pooled from three males to analyse a simple representative sample of the species via liquid chromatography tandem mass spectrometry (LC-MS/MS). (b) Cryopreservation study with and without heterospecific and conspecific seminal plasma (SP). Semen samples removed of initial seminal plasma (n = 6 replicates) and diluted with Tris extender supplemented with varying final concentrations (v/v) of good quality seminal plasma (GSP), mixed seminal plasma (MSP), and horse seminal plasma (HSP). NOSP, no seminal plasma. The Figure was created using Google Drawings.

samples as described below. Samples were held at room temperature (22–26°C) during processing, dilution, and evaluation. Semen samples were collected and processed similarly for both the proteomic analysis and cryopreservation study.

Proteomic analysis of Asian elephant seminal plasma

Sample processing for proteomic analysis

Three ejaculates each from three mature Asian elephant bulls were selected for this study. To detect the maximum range of proteins, ejaculate samples of varying initial seminal quality (0–90% fresh total motility) were chosen. Seminal plasma was separated from spermatozoa by centrifuging at 10,000g for 20 min, then aspirating the supernatant void of spermatozoa, and stored frozen (−80°C) until further analysis. On the day of proteomic analysis, SP samples were thawed on ice and centrifuged (10,000g for 5 min at 4°C) to isolate the supernatant, free of any potential spermatozoa or debris. Before proteomic analysis, total protein concentrations (mg/mL) of each SP sample were determined by bicinchoninic acid (BCA) protein quantification assay (Pierce, Rockford, IL, USA) as per the manufacturer's instructions. Bovine serum albumin was used as the protein standard. Equal volumes of each SP sample were then pooled together to create a single representative sample of the species for proteomic analysis. Equal volumes of each SP sample were used to ensure equal representation of each male and ejaculate. The pooled SP sample was kept on ice until proteomic analysis.

Liquid chromatography tandem-mass spectrometry

To identify proteins in Asian elephant SP, the pooled sample was analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) at the Bioanalytical Mass Spectrometry Facility, University of New South Wales, Australia. The liquid sample was first reduced, alkylated, and digested overnight with trypsin at 37°C. Digest peptides were separated by nano-LC using an Ultimate 3000 high performance liquid chromatography and autosampler system (Dionex, Amsterdam, Netherlands). Samples (2.5 μL) were concentrated and desalted onto a micro C18 precolumn (300 μm × 5 mm, Dionex) with H_2O:CH_3CN (98:2, 0.05% trifluoroacetic acid) at 15 μL/min. After a 4-min wash, the pre-column was switched (Valco 10 port valve, Dionex) into line with a fritless nano column (75 μm × ~10 cm) containing C18 media (1.9 μ, 120 Å, Dr Maisch, Ammerbuch-Entringen, Germany) manufactured according to Gatlin *et al.* (1998). Peptides were eluted using a linear gradient of H_2O:CH_3CN (98:2, 0.1% formic acid) to H_2O:CH_3CN (64:36, 0.1% formic acid) at 200 nL/min over 30 min. High voltage (2000 V) was applied to a low volume tee (Upchurch Scientific), and the column tip was positioned ~0.5 cm from the heated capillary (T = 275°C) of an Orbitrap Velos (Thermo Electron, Bremen, Germany) mass spectrometer. Positive ions were generated by electrospray, and the Orbitrap operated in data dependent acquisition mode (DDA).

A survey scan m/z 350–1750 was acquired in the Orbitrap (Resolution = 30,000 at m/z 400, with an accumulation target value of 1,000,000 ions) with lockmass enabled. Up to the 10 most abundant ions (>4000 counts) with charge states > +2 were sequentially isolated and fragmented within the linear ion trap using collisionally induced dissociation with an activation q = 0.25 and activation time of 30 ms at a target value of 30,000 ions. M/z ratios selected for MS/MS were dynamically excluded for 30 s.

Protein identification

All MS/MS spectra were searched against Uniprot and a customised database using MASCOT (ver. 2.4, Matrix Science, London, UK) with the following search criteria: enzyme specificity was trypsin; precursor and product ion tolerances were at 4 ppm and ±0.4 Da, respectively; variable modification of methionine oxidation; and one missed cleavage was allowed. The ions score significance threshold was set to 0.5, and each protein was provided with a probability-based MOWSE (Molecular Weight Search) score (Pappin *et al.* 1993). Ions scores were determined by −10 × log(P), where P is the probability that the observed match is a random event. Individual ion scores >20 indicated identity or extensive homology (P < 0.05). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. A higher protein score indicated a higher probability of a non-spurious match.

For identifying proteins, peptides were first searched against the completed African elephant (*Loxodonta africana*) genome (tax ID: 9784). If no match was found, then a non-restricted search was performed. To predict uncharacterised proteins in *L. africana*, FASTA codes were entered into a BLAST search restricted to the Mammalia class (tax ID: 40674).

Gene ontology

The identified proteins were further characterised for molecular functions, biological processes, cellular components, and protein classes using the PANTHER classification system (ver. 13.1; www.pantherdb.org). Gene symbols were used as input for Gene Ontology (GO) annotations for functional categorisation. To maximise the number of matched gene names and classifications of proteins, *Homo sapiens* was used as the reference species.

Evaluation of spermatozoa

Sperm motility parameters

Sperm total motility was subjectively estimated under phase contrast microscopy at ×400 magnification as described by Evans *et al.* (1987). Spermatozoa were considered non-motile if there was no flagellar movement. Simultaneously, the samples were given a kinematic rating on a scale 0–5, where 0 represented no flagellar movement and 5 represented rapid

forward progressive movement (>1 sperm length/s). For an overall sperm motility rating with equal emphasis on total motility and forward progressive motion, a sperm motility index (SMI), as described by Howard and Wildt (1990), was calculated as follows:

$$SMI = \frac{(\text{Kinematic rating} \times 20) + \text{Total motility (\%)}}{2}$$

Further motility information and sperm kinematic properties of post-thawed samples were evaluated in the laboratory using computer-assisted semen analysis (CASA; IVOS model 12.0, Hamilton–Thorne Biosciences, Beverly, MA, USA). The CASA settings and procedures have been previously described for Asian elephant spermatozoa (Thongtip *et al.* 2008). The following kinematic parameters were recorded: percentage of motile spermatozoa (MOT, %), percentage of progressively motile spermatozoa (pMOT; %), average velocity path (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH; mm), beat-cross frequency (BCF; Hz), linearity (LIN; %), and straightness (STR; %). A minimum of 300 spermatozoa over four fields of view were recorded. The CASA system could not be used for fresh and pre-freeze assessments because of the field location where semen collection and processing occurred. However, it was deemed appropriate to enhance comparison of post-thaw parameters between treatment groups.

Sperm viability and plasma membrane integrity

For assessment of viability, eosin-nigrosin stain was used as previously described (Björndahl *et al.* 2003). A minimum of 200 spermatozoa per sample were classified as viable (no stain uptake) or non-viable (partial or complete stain uptake). Viability assessments were performed within 8 h of staining and smearing. Sperm morphology was assessed on eosin-nigrosin stains by evaluating a minimum of 200 spermatozoa for abnormalities, as described by Kiso *et al.* (2013).

The functional integrity of the sperm plasma membrane was evaluated by means of the hypo-osmotic swelling test (HOST) using a modified protocol (Matson *et al.* 2009) for Asian elephant spermatozoa. A minimum of 200 spermatozoa were assessed under phase contrast microscopy (×400) for morphological changes. A positive response to hypo-osmotic stress (HOST+) resulted in spermatozoa exhibiting signs of either tail coiling or swelling to various degrees (Jeyendran *et al.* 1984), indicating normal plasma membrane integrity and function.

Acrosome integrity

The acrosome integrity of the spermatozoa was assessed by means of Coomassie blue staining technique (Larson and Miller 1999). Briefly, 10–20 µL of sample was fixed in 250 µL of 4% paraformaldehyde. A minimum of 200 stained spermatozoa per sample were evaluated for acrosomal integrity using

bright-field microscopy (×1000) under oil immersion. Spermatozoa exhibiting uniform staining over the acrosomal region were categorised as intact acrosomes, whereas those that showed non-uniform staining, abnormal shape, or lack of staining altogether in the acrosomal region were categorised as non-intact acrosomes.

Sperm DNA integrity

The integrity of sperm chromatin DNA was assessed by acridine orange (AO) fluorescence (Tejada *et al.* 1984). A minimum of 200 spermatozoa were counted on each slide by the same examiner, and the duration of evaluation per field of view did not exceed 40 s to minimise photo-bleaching effects. Spermatozoa displaying green fluorescence were considered to contain normal, intact DNA, whereas spermatozoa displaying a spectrum of yellow–orange to red fluorescence were considered to contain damaged non-intact DNA.

Cryopreservation study

Preparation of seminal plasma

Asian elephant SP was isolated from non-urine contaminated semen samples. Samples were centrifuged at 10,000g for 20 min, and then the supernatant void of spermatozoa was aspirated and stored frozen (−20°C) until used. Because of the limitations in sample availability, the SP from multiple ejaculates of Asian elephant bulls were pooled to form two SP quality groups. Ejaculates (n = 2) displaying fresh total motility ≥60% were pooled in equal volumes to form the 'good' seminal plasma (GSP) treatment. Ejaculates (n = 9) with varying fresh motility (range 0–65%) were pooled in equal volumes to form the mixed seminal plasma (MSP) treatment. A range of fresh ejaculate motility was chosen for the MSP treatment to encompass all types of ejaculate quality and better represent the 'average' Asian elephant ejaculate. Horse seminal plasma (HSP) was obtained from stallion ejaculates (n = 2) exhibiting greater than 60% fresh sperm motility. Two stallions (from Pinyopummin *et al.* 2017, 2018) were used for semen collection via artificial vagina (Davies Morel 1999). Upon pooling, semen samples were centrifuged (12,000g for 5 min) and seminal plasma aspirated. Spermatozoa from ejaculates pooled for the SP treatments (Table 1) were discarded, and new semen samples were collected for cryopreservation experiments.

Seminal plasma treatments, cryopreservation and thawing

Only ejaculates with an initial motility ≥40% were processed for cryopreservation. A lower than usual fresh motility criterion for Asian elephant semen cryopreservation (motility ≥60%; Thongtip *et al.* 2009; Kiso *et al.* 2012) was used to allow inclusion of a greater number of replicates for the purpose of statistical validity.

Fresh Asian elephant semen samples meeting this criterion (n = 6) were each diluted (1:1) with Tris-based extender

Table 1. Quality of original ejaculate samples used for seminal plasma quality groups.

	Male	Total motility (%)
Good quality seminal plasma (GSP)	Bull 1	60
	Bull 2	65
Mixed quality seminal plasma (MSP)	Bull 2	65
	Bull 3	5
		50
	Bull 4	0
	Bull 5	0
	Bull 6	40
		20
	Bull 7	0
	Bull 8	5
Horse seminal plasma (HSP)	Stallion 1	65
	Stallion 2	80

Total sperm motility of original ejaculate samples selected for seminal plasma extraction to form the seminal plasma quality groups for the cryopreservation study. Selection criteria based on total sperm motilities of fresh ejaculates or species. All samples in the table were used only for seminal plasma extraction (spermatozoa fractions were discarded).

containing 198.1 mM Tris-(hydroxymethyl)-aminomethane, 66.6 mM citric acid monohydrate, 44.4 mM glucose (Merck Millipore, USA), and 20% (v/v) egg yolk with penicillin G potassium salt (0.6 mg/mL) and streptomycin sulfate salt (1 mg/mL). This extender was adapted from use with dog spermatozoa (Peña and Linde-Forsberg 2000), and has previously shown success in preserving Asian elephant spermatozoa (Pinyopummin *et al.* 2017). All chemicals in this study were purchased from Sigma Chemical Company (St Louis, MO, USA) unless stated otherwise. The diluted samples were then centrifuged (125g for 10 min) for the removal of pre-existing SP. The sperm pellet was re-diluted with a Tris-SP mixture (from categories of SP described in Table 1) to divide each ejaculate across six treatments with final concentration of prepared SP (v/v) before freezing of: 0% SP (no seminal plasma, NOSP; as a comparative control), 12.5% GSP (GSP-low), 25% GSP (GSP-high), 12.5% MSP (MSP-low), 25% MSP (MSP-high) and 25% HSP (HSP). These concentrations were based on those used by Pinyopummin *et al.* (2017). Diluted spermatozoa were then placed into a waterbath consisting of 100 mL of water at room temperature, fully submerged, and chilled to 4°C, over 90–105 min. After chilling, samples were gradually diluted (quarter of the final volume every 15 min) with chilled Tris extender supplemented with glycerol (Bio Basic, Canada) and STM Equex paste (Nova Chemical Sales, USA) to achieve final concentrations of 5% and 0.5% (v/v), respectively. Samples were allowed to equilibrate with cryoprotectants at 4°C for approximately 15 min. Thereafter, spermatozoa were loaded into 0.5 mL straws, sealed with sealing powder, and cryopreserved by resting on a stainless-steel rack 2.5 cm above liquid nitrogen for 10 min, before being plunged and stored in liquid nitrogen until thawing (Kiso *et al.* 2012). Pre-freeze assessments were conducted after the cryoprotectant was completely added and immediately before straw loading.

Semen straws containing cryopreserved spermatozoa were thawed by agitating in a 37°C waterbath for 30 s. Spermatozoa were expelled into glass tubes and slowly diluted (1:1) with the base Tris extender warmed to 37°C. An aliquot of spermatozoa was immediately assessed for post-thaw evaluation of sperm motility, kinematics, sperm viability, plasma membrane integrity, acrosome integrity, and DNA integrity as described above (0 h). The original tube was kept at 37°C for a further 2 h and then reassessed.

Statistical analyses

Data from the cryopreservation study were analysed using Linear Mixed Model in GenStat (ver. 16, VSN International Ltd, Hemel Hempstead, UK) to determine the effects of SP treatments, time of sperm assessment, and the interaction between both. All measured sperm parameters were statistically analysed. The effects of SP treatment (GSP-low, GSP-high, MSP-low, MSP-high, NOSP and HSP) and time point (pre-freeze where applicable, and 0 h and 2 h post-thaw incubation) were included in the fixed model with bull/ejaculate as the random term. If interactions were nonsignificant they were removed from the fixed model. Data were checked for normality and homogeneity of variances before analysis. Means were compared on the basis of least significant difference and all values are reported as means $\pm$ standard error of the mean (s.e.m.). The decision was made to present the data as means $\pm$ s.e.m. despite the small sample size to enable better comparison between this study and those previously published. For all analyses, statistical significance was defined as $P < 0.05$.

Results

Proteomic analysis of Asian elephant seminal plasma

The fresh seminal characteristics of ejaculates selected for SP pooling for proteomic analysis are displayed in Table 2. Total protein concentration of Asian elephant SP ranged from 3.3 to 11.9 mg/mL with an average of 8.0 $\pm$ 1.2 mg/mL.

Mass spectrometry identified a total of 155 proteins (Supplementary Table S1) in the pooled sample of Asian elephant SP. The top 30 proteins with the highest protein scores are displayed in Table 3.

The proteins identified in Asian elephant SP are known to be involved in a wide range of molecular functions and biological processes, as per gene ontology assessment via

Table 2. Semen characteristics of selected samples pooled for proteomic analysis of Asian elephant seminal plasma.

	Ejaculate	Ejaculate fraction volume (mL)	Concentration ($\times10^6$ sperm/mL)	pH	Total motility (%)	Viability (%)	Normal morphology (%)	Seminal plasma total protein (mg/mL)
Bull 1	1	12	1280	7	0	14.2	32.1	11.7
	2	5	1322.5	6	40	10.3	46.9	8.7
	3	23	2415	6	20	8.8	66.3	11.6
Bull 2	1	5	64	8.5	80	45.3	n/a	3.3
	2	3	815	8.5	50	37.0	n/a	8.0
	3	4.5	715	9	0	62.7	57.9	9.0
Bull 3	1	3	585	8.5	90	53.0	92.9	3.6
	2	15	n/a	8	70	78.5	81.5	4.2
	3	7.5	n/a	7	50	n/a	n/a	11.9
	Mean	8.7	1028.1	7.6	44.4	38.7	62.9	8.0
	s.e.m.	±2.3	±282.1	±0.4	±10.9	±9.2	±9.1	±1.2

n/a, not applicable.

PANTHER (Fig. 2). Of the total 155 Asian elephant SP proteins, nine gene symbols could not be identified within the gene ontology database. The most common molecular functions amongst the SP protein data set were catalytic activity (GO:0003824; 54.9% of total function hits) and binding (GO:0005488; 33.6% of total function hits). In terms of biological processes, cellular (GO:0009987; 31.4% of total process hits) and metabolic (GO:0008152; 28.4% of total process hits) processes were the most common amongst the identified Asian elephant SP proteins. Only six genes (4.1% of total gene hits) were categorised with 'reproduction' as a biological process. It should be noted that a protein may be listed under more than one category. Further categorising of Asian elephant SP proteins into cellular components and protein classes can be found in Fig. S1.

Cryopreservation study

Fresh ejaculate parameters

Out of 36 attempted collections from nine bulls, 26 collections (from seven bulls) resulted in semen samples. Among these, six samples (from five bulls) met the criteria for the cryopreservation study (fresh total motility ≥40%). The fresh semen parameters of these ejaculates are summarised in Table 4.

Effect of seminal plasma of Asian elephant spermatozoa

Seminal plasma concentration. No significant effects of SP treatments were detected on total sperm motility, or on plasma membrane, acrosome and DNA integrity, both pre-freeze and post-thaw ($P > 0.05$; Table 5). The only significant effects of SP treatments on Asian elephant spermatozoa quality were detected with some sperm kinematic parameters. Sperm motility index (SMI) was higher in HSP-treated spermatozoa than in NOSP at both pre-freeze assessment and at 2 h post-thaw ($P = 0.011$; Fig. 3a). In elephant SP

treatment groups, GSP-high exhibited significantly higher SMI than NOSP at pre-freeze, while MSP-high displayed significantly higher SMI than NOSP at 2 h post-thaw. Within the same SP quality groups (GSP and MSP) there were no differences in SMI between high and low concentrations over time. Sperm kinematic metrics were only evaluated by CASA post-thaw. At 0 h post-thaw, the VAP of MSP-high treated spermatozoa was greater than that of NOSP, but by 2 h post-thaw, only HSP displayed greater VAP than NOSP ($P = 0.013$; Fig. 3b). Immediately after thawing, the VSL of MSP-low and MSP-high were significantly higher than those of NOSP ($P = 0.004$). By 2 h post-thaw, HSP and MSP-low both displayed significantly higher VSL than NOSP ($P = 0.004$; Fig. 3c). Treatments HSP and MSP-high exhibited higher BCF than NOSP at 2 h post-thaw ($P = 0.008$; Fig. 3d). Within CASA sperm parameters, no significant differences were found between high and low SP concentration within the same SP quality group (GSP and MSP; $P > 0.05$; Fig. 3). No significant effects of SP treatments were found in the other sperm kinematic parameters as measured by CASA ($P > 0.05$; Table 6).

Conspecific and heterospecific seminal plasma. No differences in sperm motility, plasma membrane integrity, and acrosome and DNA integrity were detected ($P > 0.05$; Table 5) between spermatozoa exposed to different SP quality groups (GSP and MPS) or HSP. Spermatozoa subjected to HSP displayed significantly higher SMI than those exposed to GSP-low at both pre-freeze and 2 h post-thaw ($P = 0.011$; Fig. 3a). No differences in response between exposure to Asian elephant GSP and MSP was detected with SMI evaluation ($P > 0.05$; Fig. 3a). At 2 h post-thaw, VAP was significantly higher with HSP compared to both GSP-low and GSP-high ($P = 0.013$; Fig. 3b). With Asian elephant SP, both MSP-low and MSP-high resulted in higher VAP than GSP-low

Table 3. Top 30 matched Asian elephant seminal plasma proteins as determined by LC-MS/MS from pooled samples.

UniprotKB accession	Protein name	Gene symbol	Protein mass (kDa)[A]	Protein score[B]
G3SMX8	Serum albumin	ALB	68.8	575
G3UD48	Epididymal-specific lipocalin-5	LCN5	21.5	288
G3TBR7	Low density lipoprotein receptor-related protein 2	LRP2	518.1	264
G3T8L4	Apolipoprotein D	APOD	21.6	252
G3SS80	Ribonuclease T2	RNASET2	25.5	230
G3T752	Zonadhesin	ZAN	249.2	192
G3T0S5	Lactotransferrin	LTF	77.3	188
G3TUH4	Chromosome 1 Open Reading Frame 56	C1orf56	34.1	175
G3T643	Superoxide dismutase [Cu-Zn]	SOD1	15.7	160
G3T3N6	Carboxylesterase 5A	CES5A	64.0	154
G3SZZ0	Enolase 1, (alpha)	ENO1	47.2	146
G3UCL2	Glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1	GPIHBP1	20.7	137
G3THY2	Tetraspanin (Fragment)/CD81 Molecule	CD81	23.2	134
G3U1Z4	Cathepsin D	CTSD	41.1	132
G3U2L5	Angiopoietin-Like Protein 5	ANGPTL5	26.0	121
G3UBT6	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	HSP90AA1	85.1	117
G3TZ57	Disintegrin and metalloproteinase domain-containing protein 18	ADAM18	60.9	117
G3T9G3	Transferrin	TF	108.2	111
G3SLB1	Glucosylceramidase	GBA	57.6	110
G3T8N4	Protein deglycase DJ-1	PARK7	20.0	107
G3U416	Cystatin	CST6	16.3	104
G3TBY5	Glucose-6-phosphate isomerase	GPI	62.3	104
G3SMQ4	Proteasome subunit alpha type	PSMA8	27.8	102
G3SRG6	Acrosin	ACR	40.0	102
G3SNZ3	Sperm acrosome membrane-associated protein 1	SPACA1	32.8	102
G3U7Z4	A-kinase anchor protein 4	AKAP4	89.3	99
G3T7P8	Gamma-glutamyl hydrolase	GGH	35.9	96
G3UDP9	Disintegrin and metalloproteinase domain-containing protein 21	ADAM21	79.5	95
G3UJ16	Peroxiredoxin 6	PRDX6	25.1	95
G3T7L7	Leucine-rich repeat-containing protein 37A3-like	LRRC37A3	151.3	91

[A]Protein mass as predicted from MASCOT peptide output.
[B]Ordered by descending protein score. A higher protein score indicates a higher probability of a non-spurious match from MASCOT.

by 2 h post-thaw ($P = 0.013$; Fig. 3*b*). Immediately after thawing, MSP-high and MSP-low showed higher VSL than HSP; however, by 2 h post-thaw MPS-high, GSP-high, and GSP-low all had significantly lower VSL than HSP ($P = 0.004$; Fig. 3*c*). Within elephant SP, after 2 h post-thaw MSP-low displayed higher VSL than GSP-low ($P = 0.004$; Fig. 3*c*). Sperm BCF was not different immediately at thawing, but it was higher in HSP than in GSP-low, GSP-high and MSP-low after 2 h post-thaw ($P = 0.008$; Fig. 3*d*). Within elephant SP, MSP-high demonstrated higher BCF than GSP-low ($P = 0.008$). No significant effects of SP treatments were found with the other sperm kinematic parameters as measured by CASA ($P > 0.05$; Table 6).

Discussion

This study describes two steps in the identification of possible positive effects of SP proteins on the post-thaw quality of Asian Elephant spermatozoa. The initial part provides valuable knowledge about the SP proteome of Asian elephants, while the latter component elucidates some preliminary information about the effects of differing SP profiles on Asian elephant spermatozoa during cryopreservation.

Seminal plasma proteomics provides a tool to understand the interactions of SP proteins with spermatozoa and how this may affect sperm functionality, fertilisation, and *in vitro* preservation. Determining the presence and abundance of
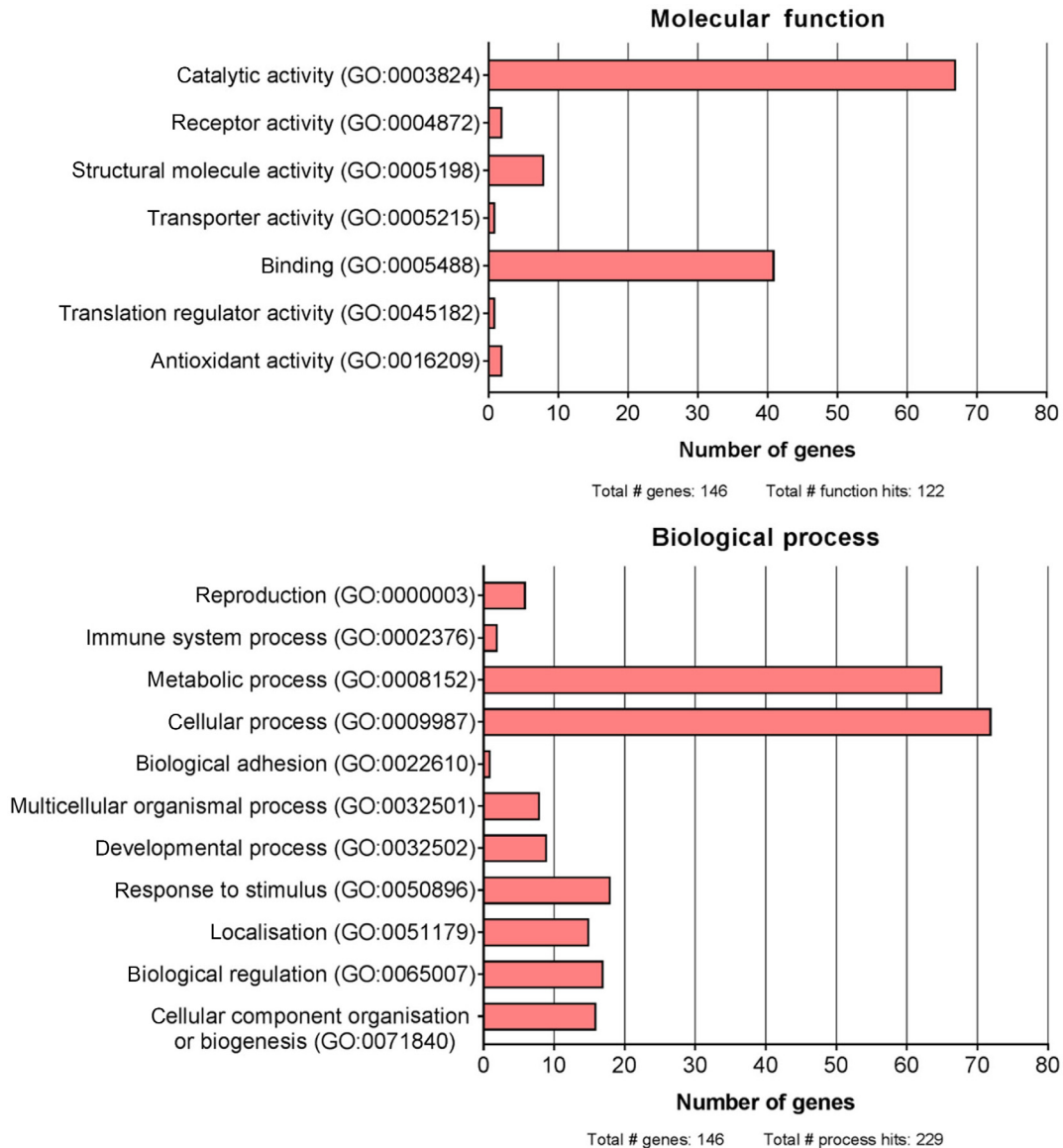
Molecular function

Total # genes: 146   Total # function hits: 122

Biological process

Total # genes: 146   Total # process hits: 229

Fig. 2. The molecular functions and biological processes of Asian elephant seminal plasma proteins. Proteins were categorised from the Gene Ontology database using PANTHER (ver. 13.1). The graph plots the number of proteins identified for each function and process (GO accession). Note: proteins can have multiple functions and processes.

selected SP proteins can be used for fertility assessment and breeding soundness of males within domestic species (Moura *et al.* 2006), or as markers of poor fertility in males (Jobim *et al.* 2011; Vilagran *et al.* 2015). Similarly, certain SP proteins have been identified in bulls with ejaculates of poor freezability (Jobim *et al.* 2004). The presence of lactotransferrin, one of the multifunctional proteins found in the current study, has been suggested to positively correlate with the fresh seminal quality of Asian elephant ejaculates (Kiso *et al.* 2013). However, the influence of SP proteins on Asian elephant spermatozoa cryotolerance and on potential differences between males remains to be elucidated.

In the current proteomic study of Asian elephant SP, 155 total proteins were matched in the UniProt databases. In another previously published study (Wattananit *et al.* 2023), 597 proteins (nearly four times more) were similarly identified in Asian elephant SP using similar tandem-mass spectrometry techniques. Greater total numbers of proteins have also been observed in other mammalian species' SP (Soleilhavoup *et al.* 2014; Perez-Patiño *et al.* 2016). However, the total number of proteins detected is dependent on the proteomic analysis techniques used and the degree of sensitivity for protein detection. The greater representation of samples and males in the proteomic analysis performed by

Table 4. Fresh semen characteristics of Asian elephant ejaculates.

Parameter	All ejaculates (seven bulls)		Ejaculates used for freezing (motility ≥ 40%; five bulls)	
	Mean ± s.e.m.	Range	Mean ± s.e.m.	Range
No. of ejaculates	26		6	
Volume (mL)	9.3 ± 1.5	(0.5–35.0)	5.1 ± 0.8	(3.0–7.5)
Sperm concentration (×10⁶/mL)	1375.1 ± 160.7	(422.5–3642.5)	1481.7 ± 337.1	(680.0–2720.0)
pH	7.4 ± 0.2	(6.0–8.5)	7.7 ± 0.3	(6.5–8.5)
Normal morphology (%)	43.2 ± 4.9	(9.3–93.5)	71.3 ± 8.7	(35.0–93.5)
Motility (%)	24.8 ± 4.0	(0.0–75.0)	57.5 ± 4.6	(40.0–75.0)
Kinematic rating (0–5)	2.8 ± 0.3	(0.0–4.5)	3.9 ± 0.3	(2.5–4.5)
Sperm motility index	40.5 ± 4.3	(0.0–82.5)	67.9 ± 5.2	(45.0–82.5)
Viability (%)	28.8 ± 4.6	(1.0–86.5)	56.8 ± 11.1	(15.5–86.5)
HOST positive (%)[A]	17.2 ± 3.2	(1.0–56.5)	35.7 ± 7.7	(5.5–56.5)
Intact acrosomes (%)	30.1 ± 4.7	(4.0–89.0)	59.0 ± 9.8	(28.5–89.0)
Intact DNA (%)	45.3 ± 5.4	(1.5–92.0)	70.3 ± 8.7	(35.0–92.0)

[A]Hypo-osmotic swelling test for plasma membrane integrity.

Table 5. *In vitro* parameters of Asian elephant spermatozoa throughout the cryopreservation process with varied presence of seminal plasma.

	Sperm parameters				
	Total motility (%)[A]	Viability (%)	HOST positive (%)	Intact acrosomes (%)	Intact DNA (%)
Pre-freeze					
NOSP	28.3 ± 6.9	47.3 ± 13.5	30.2 ± 7.6	52.3 ± 8.6	56.8 ± 9.2
GSP-low	33.3 ± 6.9	49.0 ± 13.0	30.9 ± 7.3	51.7 ± 8.5	56.9 ± 10.7
GSP-high	39.2 ± 5.7	49.8 ± 13.5	36.3 ± 8.1	51.7 ± 8.9	58.1 ± 10.2
MSP-low	32.5 ± 7.3	52.1 ± 14.6	31.6 ± 7.0	50.3 ± 8.0	56.3 ± 11.1
MSP-high	38.3 ± 5.6	50.0 ± 13.2	33.8 ± 7.5	51.6 ± 8.6	54.3 ± 8.8
HSP	40.8 ± 6.4	50.0 ± 12.8	32.1 ± 6.8	52.8 ± 7.6	58.9 ± 9.9
0 h post-thaw					
NOSP	15.8 ± 6.6	37.2 ± 8.0	14.2 ± 2.0	44.3 ± 8.2	57.9 ± 12.1
GSP-low	15.8 ± 6.6	35.9 ± 7.9	12.0 ± 1.7	44.4 ± 7.9	47.3 ± 9.9
GSP-high	19.2 ± 8.2	35.4 ± 7.6	13.0 ± 2.1	44.1 ± 8.1	47.9 ± 9.1
MSP-low	17.5 ± 5.9	36.1 ± 8.0	12.4 ± 2.1	48.3 ± 8.5	51.5 ± 10.6
MSP-high	16.7 ± 7.5	37.1 ± 7.8	14.8 ± 2.1	42.9 ± 8.8	48.6 ± 10.0
HSP	13.3 ± 5.4	33.6 ± 8.5	13.3 ± 1.9	46.0 ± 9.4	54.7 ± 8.8
2 h post-thaw					
NOSP	5.0 ± 3.2	32.1 ± 7.1	10.4 ± 1.4	41.4 ± 8.4	37.7 ± 8.0
GSP-low	7.5 ± 4.2	34.9 ± 7.5	9.2 ± 1.2	45.2 ± 8.1	39.6 ± 8.4
GSP-high	10.0 ± 6.5	37.7 ± 8.9	10.5 ± 1.9	40.8 ± 8.7	41.2 ± 9.6
MSP-low	11.7 ± 6.1	35.0 ± 7.9	10.4 ± 1.3	41.2 ± 8.6	41.8 ± 8.7
MSP-high	10.8 ± 5.4	31.8 ± 6.5	11.2 ± 0.7	44.4 ± 9.7	41.8 ± 8.1
HSP	12.5 ± 4.4	32.9 ± 7.2	13.2 ± 1.5	47.7 ± 9.2	42.8 ± 8.7

The effects of the addition of 'good' seminal plasma (GSP) and mixed seminal plasma (MSP), at high or low concentrations, absence of seminal plasma (NOSP), and horse seminal plasma (HSP) on Asian elephant sperm parameters before and after cryopreservation. Values are presented as mean ± s.e.m. Within the parameters presented, there were no significant differences between seminal plasma treatments (*P* > 0.05).
[A]Pre-freeze total motility was assessed subjectively. Post-thaw motility was assessed objectively using CASA.
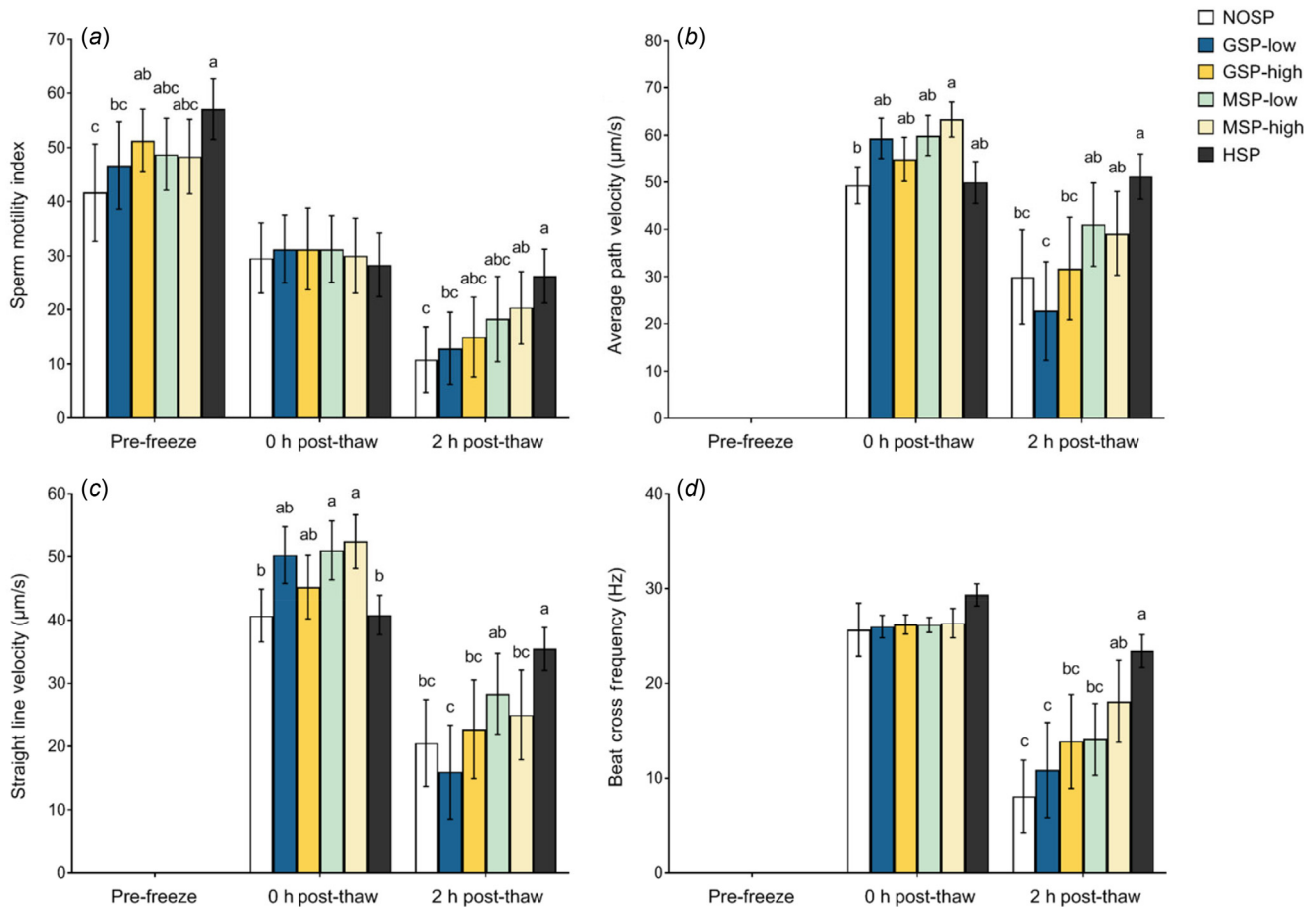
Fig. 3. Sperm motility and kinematic parameters of frozen-thawed Asian elephant spermatozoa. The effects of the absence of seminal plasma (NOSP), 'good' conspecific seminal plasma (GSP), and mixed conspecific seminal plasma (MSP), at low (12.5%, v/v) or high (25%) concentration, and horse seminal plasma (HSP 25%) on Asian elephant spermatozoa before and after cryopreservation. Data are shown as mean ± s.e.m. of (*a*) sperm motility index, (*b*) average path velocity (VAP), (*c*) straight line velocity (VSL), and (*d*) sperm head beat cross frequency (BCF). Sperm kinematic parameters assessed by CASA at post-thaw only. Within time points on each graph, different lowercase letters between treatment groups represent significant differences ($P < 0.05$).

Wattananit *et al.* (2023) may have also determined the number of detected proteins, suggesting the uniqueness of males and sample quality.

An interesting similarity between Wattananit *et al.* (2023) and this study was the relatively low proportion of mapped proteins recognised in reproductive processes (29 and 6, respectively). This emphasises the diverse, multifunctional, and ubiquitous nature of most proteins found in SP, and the great difficulty in identifying single proteins that correlate with and protect sperm parameters. The protein identification process in both studies differed. Whereas our study had peptides searched against the completed African elephant (*L. africana*) genome first, Wattananit *et al.* (2023) searched against a broader mammal protein database, likely yielding different proteins. Regardless, Asian elephant SP proteins identified by both studies have been matched with SP proteins from other species (Druart *et al.* 2013), but inter- and intraspecies comparisons show that most of them differ in

terms of isoforms or subunits. To maximise the identification of proteins in Asian elephant SP, ejaculates with varying sperm parameter qualities were included in our analysis. Through gene ontology evaluation, we observed a large variety of proteins and protein groups, reflected in a diverse array of molecular functions, protein classes, and associated biological processes. One notable group of proteins identified in Asian elephant SP is the heat shock family proteins, including HSP90AA1, HSP90B1, HSPA1A, HSPA5, HSPA4L, and HSPA9. In other species, these proteins have been shown to have beneficial effects, including aspects of reproduction such as improving the viability of ram spermatozoa (Lloyd *et al.* 2012). Additionally, they have been correlated with high sperm motility, normal morphology, and viability, and have been used as predictors of freezability in boar spermatozoa (Turba *et al.* 2007; Casas *et al.* 2009). Spermadhesins are another group of proteins that can make up a large proportion of all SP proteins in other species. Their multifunctional

Table 6. Post-thaw kinematic effects of Asian elephant spermatozoa.

	Sperm kinematic parameters					
	Motility (%)	pMOT (%)	VCL (µm/s)	ALH (µm)	STR (%)	LIN (%)
0 h post-thaw						
NOSP	11.5 ± 4.1	1.8 ± 0.9	78.5 ± 4.0	5.0 ± 1.2	79.5 ± 3.5	52.2 ± 4.7
GSP-low	10.3 ± 3.0	2.5 ± 1.1	89.6 ± 3.1	5.2 ± 0.6	82.0 ± 1.9	56.3 ± 4.4
GSP-high	14.5 ± 6.4	3.3 ± 1.9	84.3 ± 4.6	3.5 ± 0.8	76.0 ± 3.7	54.2 ± 4.1
MSP-low	14.0 ± 4.0	2.0 ± 0.9	91.4 ± 3.8	5.3 ± 0.5	82.8 ± 2.3	57.2 ± 4.8
MSP-high	12.8 ± 4.1	3.2 ± 1.4	100.6 ± 2.3	5.5 ± 0.4	78.8 ± 2.0	52.2 ± 3.8
HSP	10.2 ± 3.3	1.3 ± 0.5	83.3 ± 9.1	6.0 ± 0.4	78.3 ± 3.5	51.7 ± 3.1
2 h post-thaw						
NOSP	4.5 ± 2.6	0.2 ± 0.2	53.1 ± 18.5	1.8 ± 1.2	45.0 ± 14.5	26.5 ± 8.7
GSP-low	6.3 ± 3.3	0.3 ± 0.2	42.9 ± 20.1	3.8 ± 1.7	33.7 ± 15.1	19.2 ± 8.6
GSP-high	7.2 ± 4.1	0.7 ± 0.5	62.6 ± 20.9	3.8 ± 1.7	46.7 ± 14.8	24.5 ± 7.8
MSP-low	7.8 ± 4.0	0.5 ± 0.3	74.1 ± 16.6	3.1 ± 1.4	56.7 ± 11.5	32.5 ± 6.6
MSP-high	9.5 ± 4.8	0.8 ± 0.7	76.1 ± 16.7	3.7 ± 1.7	51.0 ± 11.6	27.2 ± 6.4
HSP	11.0 ± 4.1	0.8 ± 0.4	95.5 ± 9.2	5.2 ± 1.4	68.5 ± 2.5	39.5 ± 1.3

The post-thaw effects of 'good' seminal plasma (GSP) and mixed seminal plasma (MSP), at high or low concentration, absence of seminal plasma (NOSP), and horse seminal plasma (HSP) on Asian elephant sperm kinematic parameters as assessed by CASA. Within the parameters presented, there were no significant differences between seminal plasma treatments ($P > 0.05$).
pMOT, progressive motility; VCL, curvilinear velocity; ALH, amplitude of lateral sperm head displacement; STR, straightness; LIN, linearity.

roles include aspects of reproduction such as different stages of fertilisation (Töpfer-Petersen *et al.* 1998). Studies have suggested that spermadhesins, which are associated with binding to the sperm surface, also play a role in protecting ram spermatozoa against cold shock (Barrios *et al.* 2005). However, the specific roles of spermadhesins and many other identified proteins in Asian elephant SP remain undetermined. While functions can be surmised from studies in other species, these roles may be species-specific and not directly translatable. Furthermore, as aforementioned, proteins can be expressed as different isoforms or subunits across species (Perez-Patino *et al.* 2018; Wattananit *et al.* 2023). Mass spectrometry analysis allows for a deeper exploration of the proteomic landscape of Asian elephant SP, which may advance assisted reproductive technologies in the species and enhance our comprehension of male elephant fertility.

The second component of this study aimed to understand the effect of SP proteins on spermatozoa during the cryopreservation process. It appears that there is no consistent freezing protocol for Asian elephant spermatozoa, including whether to remove or dilute seminal plasma, despite several other publications in the area (Saragusty *et al.* 2009; Kiso *et al.* 2012; Arnold *et al.* 2017). This sort of preliminary cryopreservation research is warranted as it may help improve the post-thaw quality and fertility of Asian elephant spermatozoa. This is necessary due to the low success rate of AI with frozen-thawed spermatozoa (Thongtip *et al.* 2009) and the generally poor sperm cryosurvival observed across most males and ejaculates (Buranaamnuay *et al.* 2013; Imrat *et al.* 2013; Arnold *et al.* 2017).

Sperm velocity and kinematic characteristics, as determined by CASA, can provide valuable information about sperm activation and fertilising potential (Farrell *et al.* 1998). While this study was limited by the inability to undertake CASA assessment of the spermatozoa prior to freezing, there was still value in the ability to compare these parameters between the different treatment groups post-thaw. In the present study, total sperm motility and membrane-associated parameters did not differ between SP treatments, but some sperm kinematic parameters (CASA) did. The presence of SP resulted in elevated kinematics post-thaw when compared to the absence of SP. This finding is consistent with the corresponding patterns with SMI, which integrates subjective assessment of sperm forward progressive movement and total motility. Sperm velocity parameters VAP and VSL have been found to have a strong correlation with post-thawed bull sperm fertility (Nagy *et al.* 2015), suggesting that straight line speed may predict the likelihood of spermatozoa reaching the site of fertilisation. However, this data only applies to the motile spermatozoa, which made up only a small proportion of the total sperm population after cryopreservation in Asian elephant. The low total motility makes it problematic to extrapolate these findings for Asian elephants with confidence, and thus further work is warranted to improve the post-thaw total motility.

Within the same quality type group, the concentration of SP during the freezing process showed no difference. However higher concentration of SP (25% v/v) displayed greater sperm kinematics when compared to the absence of SP. Interestingly, the current study did not show a difference

in the cryoprotective capabilities with the addition of SP from a pool of high motility ejaculates compared the absence of SP. Our assumption that the high motility samples would contain SP components which would enhance or protect spermatozoa was not confirmed. Similar findings have been documented with horse spermatozoa whereby SP had no effect on spermatozoa post-thaw even when sourced from males with good sperm cryotolerance (Al-Essawe *et al.* 2018). The difference in categorising and quantifying a 'good' sample from which to obtain SP could explain these differences between studies. There have been few studies on the direct effects of SP on the freezing and thawing of Asian elephant spermatozoa. The addition of 10% (v/v) autologous SP to Asian elephant spermatozoa post-thaw did not improve sample quality parameters (Saragusty *et al.* 2009). Despite the complexity of SP and its many potential influencing constituents, our preliminary study is the first to show the potential protective effects of SP during cooling and thawing in this species.

The potential enhancing and protective benefits of heterospecific SP on spermatozoa are often overlooked. In this study, HSP showed a positive effect on Asian elephant spermatozoa in terms of sperm velocity parameters post-thaw. These enhanced effects of HSP were also found with SMI at pre-freeze assessment. These findings coincide with previous studies reporting that HSP supplemented in the extender during chilled liquid storage of Asian elephant spermatozoa provided greater protection of sperm motility and velocities (Pinyopummin *et al.* 2017). More specifically, HSP has been shown to increase sperm velocity parameters measured by CASA with no effect on Asian elephant sperm viability and acrosomal integrity (Pinyopummin *et al.* 2018). Interestingly, the effects of HSP on stallion spermatozoa preservation can be detrimental (Jasko *et al.* 1991; Love *et al.* 2005), though they vary depending on the ejaculate and male source (Aurich *et al.* 1996). Further investigations are needed to understand the effects of individual male Asian elephants. However, given the widespread issue of inconsistent ejaculate quality in Asian elephants (Kiso *et al.* 2013), achieving this is challenging, and there may be more noticeable effects of individual samples due to the varying seminal plasma constitution between samples (Sivilaikul *et al.* 2010; Kiso *et al.* 2013).

The component in HSP that supported elephant sperm motility has not been identified. The proteomic profile of HSP is different from other domestic species (Druart *et al.* 2013) and Asian elephant SP. The three major groups of proteins identified in HSP are fibronectin type 2, cysteine-rich secretory proteins, and spermadhesins (Töpfer-Petersen *et al.* 2005). The majority of HSP proteins (70–80% of the total proteins) belong to the fibronectin group (Calvete *et al.* 1995), known for its specific interaction with the phospholipids of the sperm membrane and their heparin-binding ability (Calvete *et al.* 1994), indicating a potential role in early fertilisation processes. However, it is challenging to identify these specific causative SP components, particularly proteins,

across different species, as the predominance of each type of heparin-binding protein varies between species (Druart *et al.* 2013) as well as between different protein families mediating heparin-binding roles (Töpfer-Petersen *et al.* 2005).

Whilst this cryopreservation study was limited due to the inconsistencies in collecting high quality ejaculates from Asian elephants, the preliminary results have shown the potential benefits that SP has on the post-thaw velocities of Asian elephant spermatozoa. Conspecific SP sourced from a large variety of elephant bulls and heterospecific HSP may provide protection of sperm velocity parameters during post-thaw incubation compared to the absence of SP. Identifying the protective components in conspecific and heterospecific HSP should be a priority for future studies. The current study has also contributed to the characterisation of the Asian elephant SP proteome, a relatively new and unexplored area. Due to limited samples, we were unable to identify the differences in the proteomic profile between high and low motility samples. Therefore, additional studies are warranted which should further investigate the impacts of SP on preservation techniques of Asian elephant spermatozoa. Nonetheless, the two reported studies have served as a foundational steppingstone in understanding Asian elephant SP and its potential role in improved sperm cryopreservation. Ultimately, further investigations in this field may aid in advancing reproductive technologies for this endangered species.

Supplementary material

Supplementary material is available online.

References

Al-Essawe EM, Wallgren M, Wulf M, Aurich C, Macias-Garcia B, Sjunnesson Y, Morrell JM (2018) Seminal plasma influences the fertilizing potential of cryopreserved stallion sperm. *Theriogenology* **115**, 99–107. doi:10.1016/j.theriogenology.2018.04.021

Aquino-Cortez A, Pinheiro BQ, Lima DBC, Silva HVR, Mota-Filho AC, Martins JAM, Rodriguez-Villamil P, Moura AA, Silva LDM (2017) Proteomic characterization of canine seminal plasma. *Theriogenology* **95**, 178–186. doi:10.1016/j.theriogenology.2017.03.016

Arnold DM, Gray C, Roth TL, Mitchell S, Graham LH (2017) A simple, field-friendly technique for cryopreserving semen from Asian elephants (*Elephas maximus*). *Animal Reproduction Science* **182**, 84–94. doi:10.1016/j.anireprosci.2017.05.003

Aurich JE, Kühne A, Hoppe H, Aurich C (1996) Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. *Theriogenology* **46**(5), 791–797. doi:10.1016/S0093-691X(96)00237-3

Azerêdo GA, Esper CR, Resende KT (2001) Evaluation of plasma membrane integrity of frozen–thawed goat spermatozoa with or without seminal plasma. *Small Ruminant Research* **41**(3), 257–263. doi:10.1016/S0921-4488(01)00189-4

Barrios B, Fernández-Juan M, Muiño-Blanco T, Cebrián-Pérez JA (2005) Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. *Journal of Andrology* **26**(4), 539–549. doi:10.2164/jandrol.04172

Ben WX, Fu MT, Mao LK, Ming ZW, Xiong WW (1997) Effects of various concentrations of native seminal plasma in cryoprotectant on viability

of human sperm. *Archives of Andrology* **39**(3), 211–216. doi:10.3109/01485019708987918

Björndahl L, Söderlund I, Kvist U (2003) Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Human Reproduction* **18**(4), 813–816. doi:10.1093/humrep/deg199

Buranaamnuay K, Mahasawangkul S, Saikhun K (2013) The *in vitro* quality of frozen-thawed Asian elephant (*Elephas maximus*) spermatozoa in semen supplemented with Equex STM paste and oxytocin during and after cryopreservation. *Reproductive Biology* **13**(2), 169–171. doi:10.1016/j.repbio.2013.04.004

Calvete JJ, Nessau S, Mann K, Sanz L, Sieme H, Klug E, Töpfer-Petersen E (1994) Isolation and biochemical characterization of stallion seminal-plasma proteins. *Reproduction in Domestic Animals* **29**(5), 411–426. doi:10.1111/j.1439-0531.1994.tb00588.x

Calvete JJ, Mann K, Schäfer W, Sanz L, Reinert M, Nessau S, Raida M, Töpfer-Petersen E (1995) Amino acid sequence of HSP-1, a major protein of stallion seminal plasma: effect of glycosylation on its heparin- and gelatin-binding capabilities. *Biochemical Journal* **310**(2), 615–622. doi:10.1042/bj3100615

Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M, Bonet S (2009) Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. *Theriogenology* **72**(7), 930–948. doi:10.1016/j.theriogenology.2009.07.001

Davies Morel MCG (1999) 'Equine artificial insemination.' (CABI Publishing: Wallingford, Oxon, UK)

Druart X, Rickard JP, Mactier S, Kohnke PL, Kershaw-Young CM, Bathgate R, Gibb Z, Crossett B, Tsikis G, Labas V, Harichaux G, Grupen CG, de Graaf SP (2013) Proteomic characterization and cross species comparison of mammalian seminal plasma. *Journal of Proteomics* **91**, 13–22. doi:10.1016/j.jprot.2013.05.029

Evans G, Maxwell WMC, Salamon S (1987) 'Salamon's artificial insemination of sheep and goats.' (Butterworths: Sydney)

Farrell PB, Presicce GA, Brockett CC, Foote RH (1998) Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology* **49**(4), 871–879. doi:10.1016/S0093-691X(98)00036-3

Flint M, McAlister DA, Agarwal A, Du Plessis SS (2015) Male accessory sex glands: structure and function. In 'Mammalian endocrinology and male reproductive biology'. (Ed. SK Singh) pp. 246–257. (CRC Press: New York, NY, USA)

Gatlin CL, Kleemann GR, Hays LG, Link AJ, Yates JR III (1998) Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography–microspray and nanospray mass spectrometry. *Analytical Biochemistry* **263**(1), 93–101. doi:10.1006/abio.1998.2809

Henault MA, Killian GJ (1996) Effect of homologous and heterologous seminal plasma on the fertilizing ability of ejaculated bull spermatozoa assessed by penetration of zona-free bovine oocytes. *Journal of Reproduction and Fertility* **108**(2), 199–204. doi:10.1530/jrf.0.1080199

Hernández M, Roca J, Calvete JJ, Sanz L, Muiño-Blanco T, Cebrián-Pérez JA, Vázquez JM, Martínez EA (2007) Cryosurvival and *in vitro* fertilizing capacity postthaw is improved when boar spermatozoa are frozen in the presence of seminal plasma from good freezer boars. *Journal of Andrology* **28**(5), 689–697. doi:10.2164/jandrol.107.002725

Hildebrandt TB, Hermes R, Pratt NC, Fritsch G, Blottner S, Schmitt DL, Ratanakorn P, Brown JL, Rietschel W, Göritz F (2000) Ultrasonography of the urogenital tract in elephants (*Loxodonta africana* and *Elephas maximus*): an important tool for assessing male reproductive function. *Zoo Biology* **19**(5), 333–345. doi:10.1002/1098-2361(2000)19:5<333::AID-ZOO5>3.0.CO;2-A

Hildebrandt TB, Göritz F, Hermes R, Reid C, Dehnhard M, Brown JL (2006) Aspects of the reproductive biology and breeding management of Asian and African elephants *Elephas maximus* and *Loxodonta africana*. *International Zoo Yearbook* **40**(1), 20–40. doi:10.1111/j.1748-1090.2006.00020.x

Howard J, Wildt DE (1990) Ejaculate-hormonal traits in the leopard cat (*Felis bengalensis*) and sperm function as measured by *in vitro* penetration of zona-free hamster ova and zona-intact domestic cat oocytes. *Molecular Reproduction & Development* **26**(2), 163–174. doi:10.1002/mrd.1080260211

Imrat P, Suthanmapinanth P, Saikhun K, Mahasawangkul S, Sostaric E, Sombutputorn P, Jansittiwate S, Thongtip N, Pinyopummin A,

Colenbrander B, Holt WV, Stout TAE (2013) Effect of pre-freeze semen quality, extender and cryoprotectant on the post-thaw quality of Asian elephant (*Elephas maximus indicus*) semen. *Cryobiology* **66**(1), 52–59. doi:10.1016/j.cryobiol.2012.11.003

Jasko DJ, Moran DM, Farlin ME, Squires EL (1991) Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. *Theriogenology* **35**(6), 1059–1067. doi:10.1016/0093-691X(91)90354-G

Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD (1984) Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility* **70**(1), 219–228. doi:10.1530/jrf.0.0700219

Jobim MIM, Oberst ER, Salbego CG, Souza DO, Wald VB, Tramontina F, Mattos RC (2004) Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. *Theriogenology* **61**(2-3), 255–266. doi:10.1016/S0093-691X(03)00230-9

Jobim MIM, Trein C, Zirkler H, Gregory RM, Sieme H, Mattos RC (2011) Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their relation with semen freezability. *Theriogenology* **76**(4), 765–771. doi:10.1016/j.theriogenology.2011.04.010

Juyena NS, Stelletta C (2012) Seminal plasma: an essential attribute to spermatozoa. *Journal of Andrology* **33**(4), 536–551. doi:10.2164/jandrol.110.012583

Kawano N, Shimada M, Terada T (2004) Motility and penetration competence of frozen-thawed miniature pig spermatozoa are substantially altered by exposure to seminal plasma before freezing. *Theriogenology* **61**(2-3), 351–364. doi:10.1016/S0093-691X(03)00218-8

Kiso WK, Asano A, Travis AJ, Schmitt DL, Brown JL, Pukazhenthi BS (2012) Pretreatment of Asian elephant (*Elephas maximus*) spermatozoa with cholesterol-loaded cyclodextrins and glycerol addition at 4°C improves cryosurvival. *Reproduction, Fertility and Development* **24**(8), 1134–1142. doi:10.1071/RD11266

Kiso WK, Selvaraj V, Nagashima J, Asano A, Brown JL, Schmitt DL, Leszyk J, Travis AJ, Pukazhenthi BS (2013) Lactotransferrin in Asian elephant (*Elephas maximus*) seminal plasma correlates with semen quality. *PLoS ONE* **8**(8), e71033. doi:10.1371/journal.pone.0071033

Larson JL, Miller DJ (1999) Simple histochemical stain for acrosomes on sperm from several species. *Molecular Reproduction & Development* **52**(4), 445–449. doi:10.1002/(SICI)1098-2795(199904)52:4<445::AID-MRD14>3.0.CO;2-6

Lloyd RE, Fazeli A, Watson PF, Holt WV (2012) The oviducal protein, heat-shock 70-kDa protein 8, improves the long-term survival of ram spermatozoa during storage at 17°C in a commercial extender. *Reproduction, Fertility and Development* **24**(4), 543–549. doi:10.1071/RD11173

Love CC, Brinsko SP, Rigby SL, Thompson JA, Blanchard TL, Varner DD (2005) Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. *Theriogenology* **63**(6), 1584–1591. doi:10.1016/j.theriogenology.2004.05.030

Martínez-Pastor F, Anel L, Guerra C, Álvarez M, Soler AJ, Garde JJ, Chamorro C, de Paz P (2006) Seminal plasma improves cryopreservation of Iberian red deer epididymal sperm. *Theriogenology* **66**(8), 1847–1856. doi:10.1016/j.theriogenology.2006.04.036

Mataveia GA, Terblanche SJ, Nothling JO, Gerber D (2010) Effect of heterologous seminal plasma and semen extenders on motility of frozen-thawed ram spermatozoa. *Journal of the South African Veterinary Association* **81**(3), 139–142. doi:10.4102/jsava.v81i3.135

Matson P, Kappelle W, Malecki I (2009) The use of a hypo-osmotic swelling (HOS) test on sperm of the pig (*Sus scrofa domesticus*), emu (*Dromaius novaehollandiae*), Asian elephant (*Elephas maximus*), hamadryas baboon (*Papio hamadryas hamadryas*), and central rock rat (*Zyzomys pedunculatus*). *Reproductive Biology* **9**(2), 181–187. doi:10.1016/S1642-431X(12)60025-4

Mogielnicka-Brzozowska M, Kordan W (2011) Characteristics of selected seminal plasma proteins and their application in the improvement of the reproductive processes in mammals. *Polish Journal of Veterinary Sciences* **14**(3), 489–499. doi:10.2478/v10181-011-0074-z

Moore AI, Squires EL, Graham JK (2005) Effect of seminal plasma on the cryopreservation of equine spermatozoa. *Theriogenology* **63**(9), 2372–2381. doi:10.1016/j.theriogenology.2004.05.032

Moura AA, Koc H, Chapman DA, Killian GJ (2006) Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. *Journal of Andrology* **27**(2), 201–211. doi:10.2164/jandrol.05089

Nagy Á, Polichronopoulos T, Gáspárdy A, Solti L, Cseh S (2015) Correlation between bull fertility and sperm cell velocity parameters generated by computer-assisted semen analysis. *Acta Veterinaria Hungarica* **63**(3), 370–381. doi:10.1556/004.2015.035

Negus C (2021) Liquid and frozen storage of Asian elephant spermatozoa. PhD Thesis, The University of Sydney, NSW, Australia.

O'Brien JK, Steinman KJ, Montano GA, Love CC, Saiers RL, Robeck TR (2013) Characteristics of high-quality Asian elephant (*Elephas maximus*) ejaculates and *in vitro* sperm quality after prolonged chilled storage and directional freezing. *Reproduction, Fertility and Development* **25**(5), 790–797. doi:10.1071/RD12129

Pappin DJC, Hojrup P, Bleasby AJ (1993) Rapid identification of proteins by peptide-mass fingerprinting. *Current Biology* **3**(6), 327–332. doi:10.1016/0960-9822(93)90195-T

Perez-Patiño C, Barranco I, Parrilla I, Valero ML, Martinez EA, Rodriguez-Martinez H, Roca J (2016) Characterization of the porcine seminal plasma proteome comparing ejaculate portions. *Journal of Proteomics* **142**, 15–23. doi:10.1016/j.jprot.2016.04.026

Peña A, Linde-Forsberg C (2000) Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. *Theriogenology* **54**(6), 859–875. doi:10.1016/S0093-691X(00)00397-6

Perez-Patino C, Parrilla I, Barranco I, Vergara-Barberán M, Simo-Alfonso EF, Herrero-Martínez JM, Rodriguez-Martínez H, Martínez EA, Roca J (2018) New in-depth analytical approach of the porcine seminal plasma proteome reveals potential fertility biomarkers. *Journal of Proteome Research* **17**(3), 1065–1076. doi:10.1021/acs.jproteome.7b00728

Pinyopummin A, Mahasawangkul S, Kornkaewrat K, Rattanapirom S, Leartsang W, Kitkha S (2017) The presence of seminal plasma, especially derived from stallion semen, helps preserve chilled Asian elephant (*Elephas maximus*) sperm motility. *Andrologia* **49**(6), e12690. doi:10.1111/and.12690

Pinyopummin A, Mahasawangkul S, Nunklang G, Kornkaewrat K, Laopiem S, Koonjaenak S, Wattananit P (2018) Supplemented stallion seminal plasma can improve impaired motility due to the dilution effect in chilled Asian elephant sperm. *Reproduction in Domestic Animals* **53**(2), 525–533. doi:10.1111/rda.13141

Pozor MA, McDonnell SM (2002) Ultrasonographic measurements of accessory sex glands, ampullae, and urethra of normal stallions of various size types. *Theriogenology* **58**(7), 1425–1433. doi:10.1016/S0093-691X(02)01034-8

Rickard JP, Leahy T, Soleilhavoup C, Tsikis G, Labas V, Harichaux G, Lynch GW, Druart X, de Graaf SP (2015) The identification of proteomic markers of sperm freezing resilience in ram seminal plasma. *Journal of Proteomics* **126**, 303–311. doi:10.1016/j.jprot.2015.05.017

Rodríguez-Martínez H, Kvist U, Ernerudh J, Sanz L, Calvete JJ (2011) Seminal plasma proteins: what role do they play? *American Journal of Reproductive Immunology* **66**(S1), 11–22. doi:10.1111/j.1600-0897.2011.01033.x

Saragusty J, Hildebrandt TB, Behr B, Knieriem A, Kruse J, Hermes R (2009) Successful cryopreservation of Asian elephant (*Elephas maximus*) spermatozoa. *Animal Reproduction Science* **115**(1-4), 255–266. doi:10.1016/j.anireprosci.2008.11.010

Schmitt DL, Hildebrandt TB (1998) Manual collection and characterization of semen from Asian elephants (*Elephas maximus*). *Animal Reproduction Science* **53**(1–4), 309–314. doi:10.1016/S0378-4320(98)00120-1

Sivilaikul S, Jitprom A, Kularb A, Kornkaewrut K, Suthanmaphinuth P, Mahasawangkul S, Saikhun K, Wajjwalku W, Thongtipsiridech S (2010) Relationship between seminal and serum calcium concentration with semen quality in the Asian elephant (*Elephas maximus*). *The Thai Journal of Veterinary Medicine* **40**(3), 251–255. doi:10.56808/2985-1130.2234

Soleilhavoup C, Tsikis G, Labas V, Harichaux G, Kohnke PL, Dacheux JL, Guerin Y, Gatti JL, de Graaf SP, Druart X (2014) Ram seminal plasma proteome and its impact on liquid preservation of spermatozoa. *Journal of Proteomics* **109**, 245–260. doi:10.1016/j.jprot.2014.07.007

Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertility and Sterility* **42**(1), 87–91. doi:10.1016/S0015-0282(16)47963-X

Thongtip N, Saikhun J, Damyang M, Mahasawangkul S, Suthunmapinata P, Yindee M, Kongsila A, Angkawanish T, Jansittiwate S, Wongkalasin W, Wajjwalkul W, Kitiyanant Y, Pavasuthipaisit K, Pinyopummin A (2004) Evaluation of post-thaw Asian elephant (*Elephas maximus*) spermatozoa using flow cytometry: the effects of extender and cryoprotectant. *Theriogenology* **62**(3–4), 748–760. doi:10.1016/j.theriogenology.2003.11.021

Thongtip N, Saikhun J, Mahasawangkul S, Kornkaewrat K, Suthanmapinanh P, Pinyopummin A (2008) Effect of pentoxifylline on the motility characteristics and viability of spermatozoa in Asian elephants (*Elephas maximus*) with low semen quality. *The Thai Journal of Veterinary Medicine* **38**(3), 37–45. doi:10.56808/2985-1130.2130

Thongtip N, Mahasawangkul S, Thitaram C, Pongsopavijitr P, Kornkaewrat K, Pinyopummin A, Angkawanish T, Jansittiwate S, Rungsri R, Boonprasert K, Wongkalasin W, Homkong P, Dejchaisri S, Wajjwalku W, Saikhun K (2009) Successful artificial insemination in the Asian elephant (*Elephas maximus*) using chilled and frozen-thawed semen. *Reproductive Biology and Endocrinology* **7**(1), 75. doi:10.1186/1477-7827-7-75

Töpfer-Petersen E, Romero A, Varela PF, Ekhlasi-Hundrieser M, Dostàlovà Z, Sanz L, Calvete JJ (1998) Spermadhesins: a new protein family. Facts, hypotheses and perspectives. *Andrologia* **30**(4-5), 217–224. doi:10.1111/j.1439-0272.1998.tb01163.x

Töpfer-Petersen E, Ekhlasi-Hundrieser M, Kirchhoff C, Leeb T, Sieme H (2005) The role of stallion seminal proteins in fertilisation. *Animal Reproduction Science* **89**(1-4), 159–170. doi:10.1016/j.anireprosci.2005.06.018

Turba ME, Fantinati P, Bernardini C, Gentilini F, Bacci ML, Forni M (2007) Relationships between innovative and traditional parameters to investigate semen quality in pigs. *Animal Reproduction Science* **99**(1-2), 72–81. doi:10.1016/j.anireprosci.2006.04.045

Ustuner B, Alcay S, Toker MB, Nur Z, Gokce E, Sonat FA, Gul Z, Duman M, Ceniz C, Uslu A, Sagirkaya H, Soylu MK (2016) Effect of rainbow trout (*Oncorhynchus mykiss*) seminal plasma on the post-thaw quality of ram semen cryopreserved in a soybean lecithin-based or egg yolk-based extender. *Animal Reproduction Science* **164**, 97–104. doi:10.1016/j.anireprosci.2015.11.017

Vilagran I, Yeste M, Sancho S, Castillo J, Oliva R, Bonet S (2015) Comparative analysis of boar seminal plasma proteome from different freezability ejaculates and identification of Fibronectin 1 as sperm freezability marker. *Andrology* **3**(2), 345–356. doi:10.1111/andr.12009

Wattananit P, Yingchutrakul Y, Kornkaewrat K, Mahasawangkul S, Roytrakul S, Pinyopummin A (2023) Non-targeted proteomic analysis of Asian elephant (*Elephas maximus*) seminal plasma using an in-solution digestion technique and liquid chromatography tandem-mass spectrometry. *Frontiers in Veterinary Science* **10**, 1174078. doi:10.3389/fvets.2023.1174078

Weber JA, Geary RT, Woods GL (1990) Changes in accessory sex glands of stallions after sexual preparation and ejaculation. *Journal of the American Veterinary Medical Association* **196**(7), 1084–1089. doi:10.2460/javma.1990.196.07.1084

Williams C, Tiwari SK, Goswami VR, de Silva S, Kumar A, Baskaran N, Yoganand K, Menon V (2020) *Elephas maximus*. The IUCN red list of threatened species 2020: e.T7140A45818198. Available at https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T7140A45818198.en [accessed 24 June 2024]

Zahn FS, Papa FO, Melo CM, Brisola ML (2005) Protein profile of equine seminal plasma: correlation to semen freezability. *Animal Reproduction Science* **89**(1-4), 313–315.

Author affiliations

[A]Faculty of Science, Sydney School of Veterinary Science, The University of Sydney, Sydney, NSW, Australia.

[B]Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand.

[C]The National Elephant Institute, The Forest Industry Organization, Lampang, Thailand.

[D]Taronga Institute of Science and Learning, Dubbo, NSW, Australia.