Mass spectrometry reveals distinct proteomic profiles in high- and low-quality stallion spermatozoa

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Abstract

The horse breeding industry relies upon optimal stallion fertility. Conventional sperm assessments provide limited information regarding ejaculate quality and are not individually predictive of fertilizing potential. The aim of this study was to harness mass spectrometry to compare the proteomic profiles of high- and low-quality stallion spermatozoa, with the ultimate goal of identifying fertility biomarker candidates. Extended stallion semen ($n = 12$) was fractionated using Percoll density gradients to isolate low-quality and high-quality sperm populations. Motility and morphological assessments were carried out, and proteomic analyses was conducted using UHPLC-MS/MS. High-quality spermatozoa recorded higher total ($95.2 \pm 0.52\%$ vs $70.6 \pm 4.20\%$; $P \leq 0.001$) and progressive motilities ($43.4 \pm 3.42\%$ vs $27.3 \pm 4.32\%$; $P \leq 0.05$), and a higher proportion of morphologically normal cells ($50.2 \pm 4.34\%$ vs $38.8 \pm 2.72\%$; $P \leq 0.05$). In total, 1069 proteins were quantified by UHPLC-MS/MS, of which 22 proteins were significantly more abundant in the high-quality sperm population ($P \leq 0.05$). A-kinase anchor protein 4 (AKAP4) and Hexokinase 1 (HK1) were considered possible biomarker candidates and their differential expression was confirmed by immunoblot. Protein expression was significantly correlated with total (AKAP4 $R^2 = 0.38$, $P \leq 0.01$; HK1 $R^2 = 0.46$, $P \leq 0.001$) and progressive motilities (AKAP4 $R^2 = 0.51$, $P \leq 0.001$; HK1 $R^2 = 0.55$, $P \leq 0.01$), percentage rapid (AKAP4 $R^2 = 0.29$, $P \leq 0.05$; HK1 $R^2 = 0.58$, $P \leq 0.001$), straight-line velocity (HK1 $R^2 = 0.50$, $P \leq 0.01$) and straightness (HK1 $R^2 = 0.40$, $P \leq 0.01$). Furthermore, AKAP4 was highly susceptible to adduction by 4-hydroxynonenal (4HNE), which resulted in a global reduction in the phosphorylation profiles following capacitation. In conclusion, the proteomic profiles of high- and low-quality stallion spermatozoa differ substantially, and proteins such as AKAP4 and HK1 could serve as biomarkers of ejaculate quality.

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Introduction

The selection of stallions for breeding focuses on pedigree and racetrack success rather than heritable reproductive traits, therefore, stallions experience lower per cycle conception (PCC) rates compared to other livestock species (Colenbrander *et al.* 1993, Parlevliet & Colenbrander 1999, Morris & Allen 2002, Nath *et al.* 2010). The Thoroughbred stud book prohibits the use of assisted reproductive technologies, while also imposing an official foal birthdate of January 1st in the northern hemisphere, and August 1st in the southern hemisphere; effectively reducing the commercial breeding season to a 4-month period. Such measures mean that breeders

experience significant pressure to achieve pregnancies as close to the start of the breeding season as possible, as larger and more mature horses will reach greater success both at yearling sales and on the racetrack (Pagan *et al.* 2005, Takahashi 2015).

The result of a given breeding cannot be ascertained until approximately 14 days post-breeding or insemination. Conventional sperm assessments, such as sperm concentration, viability, motility, and morphological assessment, although valuable, provide only limited information regarding ejaculate quality, and are not entirely predictive of the fertilizing potential (Parlevliet & Colenbrander 1999, Colenbrander *et al.* 2003, Petrunkina *et al.* 2007). Moreover, our knowledge

of the underlying molecular mechanisms leading to the formation of defective spermatozoa – and therefore periods of subfertility – is lacking compared to certain other species (Park *et al.* 2012, Intasqui *et al.* 2013, Kwon *et al.* 2015a,b, Cui *et al.* 2016, Netherton *et al.* 2017).

Unravelling the events that govern a conception success or failure, and understanding the molecular cascades that regulate fertility, requires a systems biology approach utilizing high throughput omics technologies. The advent of increasingly sophisticated proteomic technologies has enabled many detailed investigations, not only into the composition of mammalian spermatozoa (Baker *et al.* 2008, Somashekar *et al.* 2015, Swegen *et al.* 2015, Sergeant *et al.* 2016, Martín-Cano *et al.* 2020), but also into the identification of numerous proteomic fertility biomarker candidates in mice (Baker *et al.* 2005), men (de Mateo *et al.* 2007, Netherton *et al.* 2017), bulls (Park *et al.* 2012, Somashekar *et al.* 2017) and boars (Kwon *et al.* 2015a,b). To our knowledge, only one study has utilized omics technologies in identifying such biomarkers in stallion ejaculates (Novak *et al.* 2010), and reported that sperm protein expression accounted for only 10% of fertility variability. This study analyzed the full complement of cells within ejaculates, with no selection criteria to remove the dead or indeed low-quality cells; unlikely to reach the site of fertilization. Consequently, it remains unclear how a high-quality and high functioning spermatozoon, capable of fertilization, differs in composition from a low-quality cell.

Density gradient centrifugation is currently the most effective method of isolating sperm populations of differing quality within an ejaculate. Sperm populations isolated using this technique are associated with greater yields of motile, viable, morphologically normal spermatozoa, with improved fertilizing capacity, and higher pregnancy success rates (Guerin *et al.* 1989, Punjabi *et al.* 1990, Shalika *et al.* 1995, Sharma & Agarwal 1996, Carbone *et al.* 1997, Morrell *et al.* 2011, Karamahmutoglu *et al.* 2014). This technique also enables the direct comparison of sperm parameters and molecular attributes, facilitating the identification of biomarkers of fertility (Park *et al.* 2012, Cui *et al.* 2016, Netherton *et al.* 2017).

We hypothesize that by harnessing mass spectrometry technologies to compare the proteomic profile of high-quality and low-quality spermatozoa, separated using discontinuous density gradient centrifugation, we will identify robust biomarkers of ejaculate quality.

Materials and methods

Reagents

Unless specified, research-grade chemical reagents were obtained from Sigma-Aldrich. A modified Biggers, Whitten and Whittingham (BWW) medium (Biggers *et al.* 1971) containing 95 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 25 mM NaHCO₃,

5.6 mM D-glucose, 275 mM sodium pyruvate, 3.7 µL/mL 60% sodium lactate syrup, 50 U/mL penicillin, 50 mg/mL streptomycin, 20 mM HEPES (GE Healthcare) and 0.1% (w/v) polyvinyl alcohol, with an osmolarity of 310 mOsm/kg, was utilized throughout this study. The following antibodies were purchased to characterize proteins of interest: mouse monoclonal anti-AKAP4 (clone 7E10) from 4BioDx (Lille, France), mouse monoclonal anti-Hexokinase1 (Abcam), monoclonal anti-Tubulin (Sigma-Aldrich), rabbit polyclonal anti-4HNE (HNE11-S; Alpha Diagnostic International, San Antonio, TX, USA) rabbit polyclonal anti-GAPDH antibodies (G9545; Sigma-Aldrich), monoclonal anti-phosphotyrosine (P5872; Sigma-Aldrich). Percoll, nitrocellulose membranes and enhanced chemiluminescence (ECL) detection reagents were purchased from GE Healthcare, and Tris was purchased from Astral Scientific (Taren Point, NSW, Australia). Chloroform and methanol were purchased from Fronine (Riverstone, NSW, Australia) at the highest purity available. Ultrapure water was from Fluka (Castle Hill, NSW Australia). 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from Research Organics (Cleveland, OH, USA). Dynabeads Protein G and 3,3'-dithiobis[sulfosuccinimidyl propionate] (DTSSP) were purchased from Thermo Fisher Scientific. Mini-PROTEAN TGX Precast (4–20%) Protein Gels were purchased from Bio-Rad Laboratories. Paraformaldehyde was obtained from ProSciTech (Thuringowa, Qld, Australia).

Preparation of Stallion Spermatozoa

Institutional and New South Wales State Government ethical approval was secured for the use of animal material in this study. Semen samples were collected on three occasions from four normozoospermic Shetland and Miniature crossbred pony stallions (between six and nine years of age) of proven fertility, housed on institution-approved premises. Stallions had access to native pasture 24 h a day and were provided with supplementary feed consisting of grass and lucerne hay once daily.

Semen was collected using a pony-sized Missouri or Colorado artificial vagina (Minitube Australia, Ballarat, VIC, Australia) with an inline semen filter. Ejaculates were immediately diluted (2:1, extender:semen) using EquiPlus extender (Minitube Australia) and transported to the laboratory (~1 h). On arrival at the laboratory, the extended semen was fractionated using 40%/80% Percoll gradients, as previously described by Mitchell *et al.* (2011). This technique is frequently utilized to isolate spermatozoa of differing quality – in terms of motility, morphology and DNA integrity – within an ejaculate (Aitken *et al.* 2013, Xavier *et al.* 2018). Moreover, Percoll gradients were recently used to successfully identify proteins of differential expression between high- and poor-quality spermatozoa in men (Netherton *et al.* 2017). Following centrifugation (500 g, 25 min), purified spermatozoa were recovered from the 40%/80% Percoll interface (low-quality sperm), and from the 80% pellet (high-quality sperm). Samples were washed in BWW medium (500 g, 20 min) and resuspended at a concentration of 40 × 10⁶ cells/mL in BWW, for use in all assays.

Motility analysis

Sperm motility was objectively determined using computer-assisted sperm analysis (CASA; IVOS, Hamilton Thorne, Danvers, MA), with the following settings: negative phase-contrast optics, recording rate of 60 frames/s, minimum contrast of 70, minimum cell size of 4 pixels, low-size gate of 0.17, high-size gate of 2.9, low-intensity gate of 0.6, high-intensity gate of 1.74 nonmotile head size of 10 pixels, nonmotile head intensity of 135, progressive smoothed path velocity (VAP) threshold of 50 $\mu\text{m/s}$, slow (static) cell VAP threshold of 20 $\mu\text{m/s}$, slow (static) cell straight-line velocity (VSL) threshold of 0 $\mu\text{m/s}$, and threshold straightness (STR) of 75%. Cells exhibiting a VAP of $\geq 50 \mu\text{m/s}$ and a STR of $\geq 75\%$ were considered progressive. Cells with a VAP greater than that of the mean VAP of progressive cells were considered 'rapid'. A minimum of 200 spermatozoa, in a minimum of five fields were assessed using 20 μm Leja standard count slides (Gytech, Hawthorne East, VIC, Australia) and a stage temperature of 37°C.

Morphology

Approximately 4×10^6 spermatozoa were fixed in 2% paraformaldehyde for 10 min at 4°C, washed in PBS, and stored in 0.1 M glycine in PBS. Morphology was assessed using phase-contrast microscopy (Olympus CX40) at 1000 $\times$ magnification under oil immersion. A total of 100 cells were classified per sample as either being normal or having abnormalities of the head, midpiece, tail, or proximal or distal cytoplasmic droplets, as previously described (Sieme 2009).

Measurement of sperm dimensions

Approximately 4×10^6 spermatozoa were fixed in 2% paraformaldehyde, as described previously. Cells were visualized using phase-contrast microscopy (Zeiss Axio Imager A1) at 1000 $\times$ magnification under oil immersion and the following measurements were recorded on 100 cells per sample; length of head, width of head, length of midpiece and length of tail using Olympus cellSense Standard (v2.3) software.

Cell lysis

High-quality ($n=12$) and low-quality spermatozoa ($n=12$) isolated from the ejaculates of four stallions, collected on three occasions, were snap frozen in aliquots containing 40×10^6 cells, and stored at -80°C until all samples could be assessed collectively. Approximately 80 μL of lysis buffer consisting of 1% (w/v) C7BzO [3-(4-Heptyl) phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate], 7 M urea, 2 M thiourea, and 40 mM Tris (pH 10.4) was added to a pellet of 40×10^6 spermatozoa in Eppendorf tubes, for 1 h at 4°C with constant rotation. Samples were centrifuged (18,000 g , 15 min), and supernatants were recovered and transferred to fresh tubes. Protein quantification was performed using a 2D quant kit (GE Healthcare) following the manufacturer's protocol.

Protein precipitation

Lysate was collected and reduced using 10 mM 1,4-dithiolthreitol (DTT) for 1 h at room temperature (RT) and then alkylated with 20 mM iodoacetamide (IAA) for one h at RT, in the dark. Approximately 100 μg of protein per sample were precipitated using the methanol/chloroform protocol, as described previously (Wessel & Flügge 1984). Briefly, lysates were adjusted to 400 μL with ultrapure water. Methanol and chloroform were then added to lysates at a ratio of 2:2:1 (lysate:MeOH:CHCl₃), inverted and centrifuged briefly (17,000 g , 1 min). The upper aqueous phase was removed (leaving the interfaced, protein precipitate layer intact), and 300 μL methanol was added. Lysates were gently inverted and centrifuged (17,000 g) for 15 min. Supernatants were discarded and lysates were air-dried for 15 min at RT.

In-solution trypsin digestion

Protein pellets were solubilized in 200 μL of 5M urea and mixed with 100 μL of 25 mM ammonium bicarbonate buffer (pH 8.5). Proteins were reduced by adding 30 μL of 10 mM DTT (20 min, 56°C) and alkylated by adding 30 μL of 20 mM IAA (30 min, RT) in the dark. Digestion was performed by adding 1 μL of proteomics grade trypsin (1 $\mu\text{g}/\mu\text{L}$ in trypsin 1 mM HCl) and samples were incubated overnight (37°C). The reaction was stopped using 10 μL of 0.1% formic acid, and filtering samples through 0.2 μm (hydrophilic PTFE) into a 2 mL dark glass vial. Finally, samples were dried using a nitrogen current at 35°C and resuspended in 20 μL of H₂O/acetonitrile/formic acid buffer (94.9:5:0.1).

UHPLC-MS/MS analysis

The separation and analysis of the samples were performed using the ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS); consisting of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies), equipped with an automated multi-sampler module and a high speed binary pump, and coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies), using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. The control of the UHPLC-MS and Q-TOF were made using MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01). Samples were injected onto an Agilent AdvanceBio Peptide Mapping HPLC column (2.7 μm , 150 $\times$ 2.1 mm, Agilent technologies), at 55°C, at a flow rate of 0.4 mL/min. The gradient program began with 2% of buffer B (H₂O/acetonitrile/formic acid buffer, 10:89.9:0.1) in isocratic mode (5 min), increasing linearly to 45% (40 min), followed by 95% (15 min), remaining constant for five min. Buffer A: H₂O/acetonitrile/formic acid buffer, 89.9:10:0.1. The mass spectrometer was operated in the positive mode. The nebuliser gas pressure was set to 35 PSI, drying gas flow was set to 10 L/min at a temperature of 250°C, and the sheath gas flow was set to 12 L/min at a temperature of 300°C. The capillary spray, fragmentor and octopole RF Vpp voltages were 3500, 340 and 750 V, respectively. Profile data were acquired for both MS and MS/MS scans in extended dynamic range mode. MS and MS/

MS mass range was 50–1700 m/z , and scan rates were eight spectra/sec for MS, and three spectra/s for MS/MS. Auto MS/MS mode was used with precursor selection by abundance and a maximum of 20 precursors selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of –4.8. The same ion was rejected after two consecutive scans.

Proteomic data processing and analysis

Data processing was performed using Spectrum Mill MS Proteomics Workbench (rev B.04.01, Agilent Technologies). Briefly, raw data were extracted under default conditions as follows: non fixed or variable modifications were selected; [MH]⁺ 50–10,000 m/z ; maximum precursor charge +5; retention time and m/z tolerance ± 60 s; minimum signal-to-noise MS (S/N) 25; finding ¹²C signals. The MS/MS was searched against the UniProt horse database (downloaded September 2019) and performed with the following criteria; no fixed modifications, carbamidomethylated cysteines (variable modification); tryptic digestion with a maximum of five missed cleavages; ESI-Q-TOF instrument; minimum matched peak intensity 50%; maximum ambiguous precursor charge +5; monoisotopic masses; peptide precursor mass tolerance 20 ppm; product ion mass tolerance 50 ppm; and calculation of reversed database scores. Validation of peptide and protein data was performed using auto thresholds with a false discovery rate of 1.2% and summarized using the validations; score >5 and 60% SPI (Scored Peak Intensity: the percentage of the extracted spectrum that is explained by the database search result).

Exported protein lists were imported to Perseus (version 1.6.10.43; [Tyanova et al. 2016](#)), and z-scoring normalization was performed on transformed data, followed by Student's *t*-tests. From this data, volcano plots were created between high- and low-quality populations harvested from each individual stallion, where the x-axis is the log₂ fold change and the y-axis is the –log₁₀ *P* value score ([Nixon et al. 2019b](#)). Using the refined protein list (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article), accessions were mapped to UniProt to collate information on level of protein evidence and associated Gene Ontology classifications of biological processes, molecular function and cell localization. In cases of obsolete or deleted protein entries (109 proteins), a Basic Local Alignment Search Tool (BLAST) search was carried out to identify putative genes, based on a minimum 50% shared identity (Supplementary Table 1). The initial protein list was further refined, based on the inclusion of proteins with a quantitative value in at least two technical replicates of one stallion, resulting in the identification of 499 proteins (denoted by fold change and *P*-values; Supplementary Table 1).

SDS-PAGE and immunoblotting

Candidate proteins that demonstrated a statistically significant fold-change between sperm fractions, and a spectral count >100, were identified. These proteins were cross-checked against a literature search of studies identifying fertility biomarker proteins in various species. A-kinase anchor protein

4 (AKAP4) and Hexokinase 1 (HK1) were repeated implicated ([Intasqui et al. 2013](#), [Frapsauce et al. 2014](#), [Cui et al. 2016](#), [Blommaert et al. 2019](#)) and were therefore chosen for validation by immunoblotting. All cells were boiled (100°C, 5 min) in sodium dodecylsulphate (SDS) extraction buffer (0.375 M Tris pH 6.8, 2%, w/v SDS, 10%, w/v sucrose, protease inhibitor cocktail) to extract protein. Samples were centrifuged (17,000 *g*, 15 min, 4°C) and soluble protein remaining in the supernatant was quantified using a DC protein assay kit (Bio-Rad). A total of 10 µg of protein from each sample was boiled (5 min, 100°C) in SDS-PAGE (PAGE) sample buffer (2%, v/v mercaptoethanol, 2%, w/v SDS, and 10%, w/v sucrose in 0.375 M Tris, pH 6.8, with bromophenol blue). Protein was resolved by SDS-PAGE (150 V, 1 h), transferred to nitrocellulose membranes (350 mA, 1 h), and blocked (1 h, RT) in 5% skimmed milk, or 3% BSA in Tris-buffered saline (TBS; 100 mM Tris-HCl, pH 7.6, and 150 mM NaCl) supplemented with 0.1% Tween-20 (TBST). Primary antibodies for AKAP4 (1:1000) and 4HNE (1:500) were diluted in 1% skim milk/TBST, while those for HK1 (1:1000), Tubulin (1:3000) and GAPDH (1:4000) were diluted in 1% BSA/TBST. Membranes were incubated in primary antibody overnight (4°C) under rotation and probed (1 h, RT) with appropriate horseradish peroxidase-conjugated secondary antibodies diluted in 1% skim milk/TBST or BSA/TBST (anti-rabbit 1:2500; anti-mouse 1:2000). Following washing (three times for 5 min in TBST), cross-reactive proteins were visualized using an enhanced chemiluminescence (ECL) kit, according to the manufacturer's instructions, and exposed to a digital camera LAS-3000 system (GE Healthcare). Semi-quantification was achieved using ImageJ software (NIH). Sperm SDS lysate blots were stripped and re-probed with anti-Tubulin or anti-GAPDH antibody as a loading control.

Co-immunoprecipitation

Co-immunoprecipitation (IP) was utilized to assess the covalent modification of AKAP4 by 4HNE in high and low-quality sperm populations. Spermatozoa were lysed in IP lysis buffer (10 mM CHAPS, 10 mM HEPES, 137 mM NaCl and 10% glycerol) on constant rotation (2 h, 4°C). Lysates were recovered following centrifugation (13,800 *g*, 20 min, 4°C) and 100 µL was added to 50 µL aliquots of washed Dynabeads and incubated under rotation to pre-clear (1 h, 4°C). Anti-AKAP4 antibody (10 µg in 200 µL of PBS) was conjugated to fresh aliquots of washed Dynabeads (supernatant removed) by incubation under rotation (2 h, 4°C). Cross-linking was performed by adding 3,3'-dithiobis[sulfosuccinimidyl propionate] (DTSSP) at a final concentration of 2 mM (30 min, RT), after which 2 mM Tris was added (15 min, RT) to quench the reaction. Dynabeads were then washed three times in 200 µL of lysis buffer and supernatants were retained. Immunoprecipitation was performed by adding 100 µL of lysates to AKAP4 antibody-bound beads and incubating on constant rotation overnight (4°C). Supernatants were transferred to fresh tubes and washed three times in 200 µL of PBS. Beads were resuspended in 100 µL of PBS and transferred to fresh tubes to avoid co-elution of proteins bound to the tube. Antibody-antigen-bound beads, pre-cleared beads, 10 µL of fresh protein G beads (bead-only

control), 5 μ L anti-AKAP4 (antibody-only control) and 5 μ L of the final PBS wash of the antibody bound beads (negative control) were boiled in SDS loading buffer (containing 8% β -mercaptoethanol) for 5 min to elute proteins. The eluted proteins were then loaded onto a 4–20% Tris-Glycine gel, resolved by SDS-PAGE (150 V, 1 h) transferred to nitrocellulose membranes (350 mA, 1 h) and blocked as detailed previously. The blots of eluted sperm proteins were probed with anti-AKAP4, then stripped and re-probed with anti-4HNE antibody to assess 4HNE adduction.

4HNE treatment and capacitation

4HNE was used to assess the effects of aldehyde exposure on tyrosine phosphorylation. Spermatozoa recovered from 40%/80% Percoll interface, and from the 80% pellet were incubated (30 min, 37°C) with 4HNE (0, 50 or 100 μ M), washed in BWV (500 μ L, 3 min) and resuspended in 500 μ L of BWV capacitation medium (0.5 mM methyl- β -cyclodextrin, 3.0 mM pentoxifylline, 5 mM dibutyl cyclic AMP, 3 mg BSA in 10 ml BWV; Bromfield *et al.* 2014) or normal BWV medium in flat-bottom tubes. All samples were incubated under an atmosphere of 5% CO₂ and 95% air for 4 h (37°C). Throughout the incubation period, sperm suspensions were gently inverted at 30 min intervals to prevent settling of the cells. Samples were gently centrifuged (500 μ g, 3 min), supernatant was removed, and the resulting pellet was frozen (–80°C). Immunoblotting was performed as detailed previously. Membranes were blocked (3% BSA/TBST) and then probed with anti-phosphotyrosine antibody (α -PT66) diluted in 1% BSA/TBST (1:2000) under constant rotation overnight (4°C). Membranes were washed, probed with anti-mouse HRP secondary antibody (1:2000 in 1% BSA/TBST) and visualized.

Statistical analyses

Normality checks were conducted on all data using Shapiro–Wilk test in SPSS version 25 software (IBM Corp.) and where data was not normally distributed, appropriate data transformations were performed depending on the degree and direction of skewness. T-tests and Pearson correlations were performed using SPSS v 25 software (IBM Corp.).

Results

Assessment and characterization of high- and low-quality sperm fractions

Purified poor-quality spermatozoa were recovered from the 40%/80%-Percoll interface and high-quality spermatozoa were recovered from the 80% pellet. Significant differences in motility and cell morphology were evident between high- and low-quality spermatozoa. An average of 43.4% ($\pm 3.42\%$) of the high-quality sperm fraction demonstrated forward, progressive motility, in comparison with only 27.3% ($\pm 4.32\%$) of the low-quality sperm fraction ($P \leq 0.05$; Fig. 1A). Specific morphological assessments of both

Figure 1 Total and progressive motility (A), and the percentage of morphologically normal cells (B) in high-quality (closed bar) and low-quality (open bar) sperm fractions ($n = 12$). Data correspond to mean values $\pm$ S.E.M. * $P \leq 0.05$, *** $P \leq 0.001$.

populations revealed 50.2% ($\pm 4.34\%$) of the high-quality sperm fraction were classed as morphologically normal, in comparison to 38.8% ($\pm 2.72\%$) of the low-quality sperm fraction ($P \leq 0.05$; Fig. 1B). Morphological abnormalities detected between populations are presented in Table 1.

Table 1 Proportion of morphological abnormalities present in high- (80%-Percoll fraction) and low-quality (40%-Percoll fraction) sperm populations.

Classification	High quality sperm fraction (%)	Low quality sperm fraction (%)	P-value
Morphologically normal	50.2 $\pm$ 4.34	38.8 $\pm$ 2.72	≤ 0.05
Head abnormality	17.6 $\pm$ 2.59	24.2 $\pm$ 2.97	N/S
Mid-piece abnormality	21.6 $\pm$ 3.22	21.5 $\pm$ 1.96	N/S
Tail abnormality	7.2 $\pm$ 2.27	9.5 $\pm$ 2.41	N/S
Proximal cytoplasmic droplet	1.9 $\pm$ 0.78	4.2 $\pm$ 1.05	N/S
Distal cytoplasmic droplet	1.6 $\pm$ 0.66	1.9 $\pm$ 0.59	N/S

Table 2 List of identified proteins with significantly higher abundance in high-quality Percoll-derived spermatozoa, compared to low-quality Percoll-derived spermatozoa.

Uniprot accession no.	Protein	Gene	P-value	Fold change
F6Y1A5	Phospholipase A2 receptor 1	<i>PLA2R1</i>	0.001	104.35
F7CZM1	Cytochrome c oxidase subunit 5A	<i>COX5A</i>	0.001	13.50
Q6H321	Kallikrein-1E2	<i>KLK1E2</i>	0.002	9.69
K9K2H8	Mitochondrial 3-ketoacyl-CoA thiolase-like protein	<i>ACAA2</i>	0.002	16.50
F6TCZ6	GRB2 associated regulator of MAPK1 subtype 2	<i>GAREM2</i>	0.002	195.25
F7DN04	RPTOR independent companion of MTOR complex 2	<i>RICTOR</i>	0.003	82.37
F6ZWQ8	Parkin coregulated	<i>PACRG</i>	0.005	8.32
F7ANR3	Proteasome subunit alpha type	<i>PSMA3</i>	0.014	57.75
F6RBE2	Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH5</i>	0.014	1.06
F7BUS7	TSC22 domain family member 4	<i>TSC22D4</i>	0.016	205.33
F6ZKT4	Adenylate kinase 8	<i>AK8</i>	0.016	38.32
F6Y0B1	A-kinase anchoring protein 4	<i>AKAP4</i>	0.016	1.18
F6ZMI7	Lysozyme A	<i>LYZL4</i>	0.017	4.43
F7AAD0	Sperm acrosome associated 3	<i>SPACA3</i>	0.021	2.91
F6ZD33	Septin 8	<i>SEPTIN8</i>	0.026	37.69
B3IVM0	Pyruvate kinase	<i>PKM</i>	0.028	1.71
F6WSG0	Oxoglutarate dehydrogenase	<i>OGDH</i>	0.028	29.52
F7BXF3	Histone H2A	<i>H2AX</i>	0.030	11.55
F6WY50	NADH:ubiquinone oxidoreductase subunit S4	<i>NDUFS4</i>	0.032	92.22
F7E1S0	Rho GTPase activating protein 11A	<i>ARHGAP11A</i>	0.049	428.08
F7BA85	Hexokinase 1	<i>HK1</i>	0.050	1.53
K9KEM4	ADP/ATP translocase 2-like protein	<i>SLC25A5</i>	0.051	3.39

Proteomic characterization of high- and low-quality sperm fractions

Data obtained from UHPLC-MS/MS identified 1069 proteins (Supplementary Table 1), and the abundance of 22 of these proteins were significantly higher in the high-quality Percoll-fractionated spermatozoa than in the low-quality spermatozoa (Table 2). Based on our analysis using the UniProt Equine database, of the 1069 proteins we identified, only 1% have reported evidence at protein level (Fig. 2A). Of the remaining; 66 have evidence at transcript level only; 309 are inferred from homology; while 578 are predicted, with no evidence at protein, transcript, or homology levels. A total of 109 protein entries were obsolete or deleted, and were replaced with putative genes following a BLAST search (Supplementary Table 1).

To determine the key biological processes and molecular functions involved in the development of low-quality spermatozoa, protein accessions were mapped to UniProt. Classification according to biological process (Fig. 2B) revealed the dominant categories to be those associated with positive regulation (365 proteins), negative regulation (246 proteins), and the response to stimuli (187 proteins). When the category of positive regulation was analyzed further, the most represented processes included the regulation of transcription and translation (35 proteins); the regulation of protein activity (including expression, localization and post-translational modifications – 64 proteins); and the regulation of cellular processes (including growth, differentiation and apoptosis – 33 proteins). The category of negative regulation mapped to similar

primary categories representing 33 (transcription and translation); 32 (regulation of protein activity); and 52 proteins (regulation of cellular processes), while the principal categories comprising 'response to stimuli' were; response to stress (including heat and oxidative stress – 32 proteins); pathogenic response (27 proteins); protein response (namely the folding of proteins – 12 proteins); and finally, response to DNA damage (8 proteins). Volcano plots were constructed to highlight the inter-individual variability in protein abundance between high- and low-quality cell populations collected from stallions 1–4 (Fig. 2C, D, E and F, respectively).

Immunoblotting validation

Two candidate proteins were chosen for validation by immunoblotting using the criteria of a statistically significant fold-change between sperm fractions, and a spectral count >100. These included Hexokinase 1 (HK1) and A-kinase anchor protein 4 (AKAP4). In both cases, these proteins were identified as significantly more abundant in the high-quality sperm fraction when compared to the low-quality fraction ($P \leq 0.05$). An immunoblot using the anti-HK1 antibody demonstrated a major band of ~100 kDa (Fig. 3A), which could be normalized to the loading control (Fig. 3B); only two representative blots are shown. Band density quantification of all high-quality ($n=12$) and low-quality ($n=12$) samples analyzed, confirmed our MS analysis findings (Fig. 3C). To highlight the functional significance of these results, the relationship between HK1 band density and sperm motility ($n=12$) was

Figure 2 Compositional analysis of proteomic data, and inter-stallion variability in protein expression. (A) Classification of protein existence, according to UniProt. (B) Gene Ontology annotation of biological processes. The ten most highly represented categories (highest number of assigned proteins) are shown. (C, D, E and F) Volcano plots corresponding to stallions 1–4 highlight inter-stallion variability in protein expression; x-axis ($\log_2$ fold change) and y-axis ($-\log_{10} P$ value; cut off ≥ 1.3).

Figure 3 Assessment of HK1 expression in sperm populations. (A) Cells were lysed in an SDS-based extraction buffer, resolved by SDS-PAGE and prepared for immunoblotting with anti-HK1 antibodies ($n = 12$). (B) Blots were subsequently stripped and probed with anti-Tubulin antibodies as a loading control. Representative blots of lysates from high-quality ('high') and low-quality ('low') sperm fractions are shown. (C) Band densitometry analysis of HK1 (100 kDa); mean values ($\pm$ S.E.M.) are presented relative to the corresponding Tubulin control. (D) Correlation between progressive motility and HK1 abundance. $**P \leq 0.01$.

assessed. Positive correlations existed between the HK1 abundance in high- and low-quality spermatozoa and total motility ($R^2 = 0.46$, $P \leq 0.001$), progressive motility ($R^2 = 0.55$, $P \leq 0.001$; Fig. 3D), rapid motility ($R^2 = 0.58$, $P \leq 0.001$), VSL ($R^2 = 0.50$, $P \leq 0.01$), STR ($R^2 = 0.40$, $P \leq 0.01$) and LIN ($R^2 = 0.32$, $P \leq 0.05$).

An immunoblot using the anti-AKAP4 antibody demonstrated major bands of ~ 82 and ~ 110 kDa corresponding with AKAP4 and its precursor molecule proAKAP4, respectively (Fig. 4A), which could be normalized to the loading control (Fig. 4B); only three representative blots are shown. Plotting the ~ 82 kDa AKAP4 band densities of all high-quality ($n = 12$) and low-quality ($n = 12$) samples assessed, confirmed the findings of our MS analysis ($P \leq 0.05$; Fig. 4C). ProAKAP4 abundance was also significantly higher in the high-quality compared to the low-quality sperm fraction ($P \leq 0.05$, $n = 12$). Strong, positive correlations existed between AKAP4 expression ($n = 12$) and total motility ($R^2 = 0.38$, $P \leq 0.01$), progressive motility ($R^2 = 0.49$, $P \leq 0.001$; Fig. 4D) and rapid motility ($R^2 = 0.29$, $P \leq 0.05$).

Sperm dimensions

To confirm that the underexpression of AKAP4 and HK1 in low-quality sperm fractions was not the result

Figure 4 Assessment of AKAP4 expression in sperm populations. (A) Cells were lysed in an SDS-based extraction buffer, resolved by SDS-PAGE and prepared for immunoblotting with anti-AKAP4 antibodies ($n = 12$). (B) Blots were subsequently stripped and probed with anti-Tubulin antibodies as a loading control. Representative blots of lysates from high-quality ('high') and low-quality ('low') sperm fractions are shown. (C) Band densitometry analysis of AKAP4 (82 kDa); mean values ($\pm$ S.E.M.) are presented relative to the corresponding Tubulin control. (D) Correlation between progressive motility and AKAP4 abundance. *** $P \leq 0.001$.

of sperm fragmentation or differences in cell size, we measured the mid-piece length, tail length, head length and head width of 100 cells from each sample. No significant differences were present between the head length ($7.0 \pm 0.04 \mu\text{m}$ vs $7.0 \pm 0.04 \mu\text{m}$), head width ($3.7 \pm 0.04 \mu\text{m}$ vs $3.7 \pm 0.03 \mu\text{m}$), mid-piece length ($10.4 \pm 0.06 \mu\text{m}$ vs $10.6 \pm 0.06 \mu\text{m}$), tail length ($48.0 \pm 0.25 \mu\text{m}$ vs $47.9 \pm 0.19 \mu\text{m}$), or mid-piece:tail length ratio (ratio 4.6 ± 0.03 vs 4.6 ± 0.03) of spermatozoa isolated from high- and low-quality sperm fractions.

Co-immunoprecipitation

To explore the conservation and extent of AKAP4 aldehyde adduction in stallion sperm fractions, co-immunoprecipitation was performed, whereby sperm lysates were precipitated with anti-AKAP4 antibody, and the eluates were subsequently examined for the presence of AKAP4 and 4HNE. As illustrated in Fig. 5A, this technique effectively isolated AKAP4, and following subsequent stripping and re-probing of these blots, predominant bands of 4HNE were detected at an equivalent molecular weight to AKAP4 (82 kDa), thus confirming AKAP4 as a target for 4HNE adduction in stallion spermatozoa (Fig. 5B). Moreover, densitometry analysis confirmed 4HNE adduction was significantly higher in high-quality spermatozoa (which possess a higher quantity of the AKAP4 substrate; Fig. 5A and C) than low-quality spermatozoa ($P \leq 0.05$; Fig. 5C). The inclusion of immunobead-only and antibody-only controls, together with preclear bead eluates, and bead-washes prior to elution, verified the specificity of each immunoprecipitation.

Figure 5 Analysis of 4HNE adduction to AKAP4. (A) Confirmation of AKAP4/4HNE interaction in both high-quality and low-quality sperm populations and was sought using immunoprecipitation, whereby AKAP4 was used to pull down interacting partners. The captured sperm proteins were eluted from protein G Dynabeads and resolved on SDS-PAGE gels alongside an antibody-only control (AB only), a bead-only control (bead only), sperm lysates (high-quality and low-quality fraction elutions) and precleared controls (high-quality and low-quality fraction preclears). (B) Re-probing these blots with anti-4HNE revealed heavy adduction of 4HNE to AKAP4 at 82 kDa in both elutions. (C) Band densitometry analysis of 4HNE bound to AKAP4 (82 kDa); mean values ($\pm$ S.E.M.) are presented relative to the corresponding AKAP4 band density. * $P \leq 0.05$

4HNE adduction and stallion sperm capacitation

Given that AKAP4 was heavily adducted by 4HNE in high-quality stallion spermatozoa, we extended our analysis to assess the consequences of 4HNE adduction on the ability of these cells to undergo capacitation as assessed by phosphotyrosine labelling; a key correlate of the capacitation process. Immunoblotting revealed that 4HNE treatment (both 50 and 100 μM concentrations) caused a global reduction in the tyrosine phosphorylation profile (Fig. 6A, C and D), particularly seen in bands resolving at 64–70 kDa, with significant under-expression or absence in cells which had been subject to 4HNE insult prior to capacitation (Fig. 6E).

Discussion

This is the first study to identify differentially accumulated proteins between the high- and low-quality sperm fractions of stallion ejaculates. A total of 1069 proteins were identified using UHPLC-MS/MS (Supplementary Table 1), of which 22 exhibited significantly higher abundance in the high-quality sperm fraction (Table 2). The identification of these proteins will lead to many future investigations regarding sperm function, quality, composition and overall stallion fertility. Furthermore, we have provided experimental evidence for 1063 stallion sperm proteins which were otherwise predicted, inferred or shown at transcript level only (Fig. 2A).

The differential expression of two of these proteins – Hexokinase 1 (HK1) and A-kinase anchor protein 4 (AKAP4) – was confirmed by immunoblotting analysis. Hexokinases are a family of enzymes functioning in

Figure 6 4HNE treatment attenuates capacitation-associated tyrosine phosphorylation. High-quality and low-quality sperm populations were either exposed to 4HNE (50 or 100 μ M) or untreated ('UT'), and incubated for 1 h at 37°C. Spermatozoa were induced to capacitate ('Cap') or held in a non-capacitated state ('N/C') using standard protocols. (A and B) Spermatozoa were subsequently prepared for immunoblotting with anti-phosphotyrosine antibodies and re-probed with a loading control (representative blots shown). (C and D) A densitometric trace of representative immunoblots is included to highlight changes in phospho-labelling in untreated (UT; black trace) and 50 μ M 4HNE treated (4HNE; red trace) spermatozoa isolated from the high- and low-quality Percoll fractions. (E) Densitometry analysis of bands 64–70 kDa; mean values ($\pm$ S.E.M.) are presented relative to the corresponding loading control. Mean values without a common superscript differ significantly ($P \leq 0.001$).

the first step of glucose metabolism, by catalysing the phosphorylation of glucose to glucose-6-phosphate (G6P; Wilson 2003). HK1, the most ubiquitous of the major hexokinase isoforms, is localized to the sperm midpiece and fibrous sheath, via the porin binding domain in its hydrophobic N-terminal (Polakis & Wilson 1985, Wilson 2003, Sun *et al.* 2008). HK1 has previously been implicated as a marker of fertility in men (Intasqui *et al.* 2013, Cui *et al.* 2016, Fanny *et al.* 2018). Cui *et al.* (2016) reported a significantly higher abundance of HK1 in the high-quality sperm fraction, while Intasqui *et al.* (2013) reported higher HK1 abundance in spermatozoa with low levels of DNA damage. In the present study, the functional significance of HK1 was reinforced by the existence of a strong, positive relationship between HK1 expression and numerous motility parameters (Fig. 3D); a result that is not surprising considering the involvement of HK1 in energy metabolism (Wilson 2003).

In spermatozoa, AKAP4 is the most abundant member of the AKAP family – a conserved dynasty of diverse scaffolding proteins – which conduct pivotal roles in fibrous sheath formation, motility, capacitation and acrosome reaction, while orchestrating the integration of cAMP and various components of signal-transduction pathways (Colledge & Scott 1999, Moss *et al.* 1999, Miki *et al.* 2002, Brown *et al.* 2003, Welch *et al.* 2010, Luconi

et al. 2011, Langeberg & Scott 2015, Rahamim Ben-Navi *et al.* 2016). Localized to the principal piece of the sperm flagellum, AKAP4 accounts for almost 50% of all fibrous sheath proteins (Carrera *et al.* 1994, Eddy *et al.* 2003, Krisfalusi *et al.* 2006). Considering the vital roles played by AKAP, it is not surprising that its underexpression has been repeatedly implicated in infertility in men (Xu *et al.* 2012, Frapsauce *et al.* 2014) bulls (Peddinti *et al.* 2008, Legare *et al.* 2017, Singh *et al.* 2019) and stallions (Blommaert *et al.* 2019). AKAP4 is synthesized from its precursor molecule proAKAP4, (Delehedde *et al.* 2019, Sergeant *et al.* 2019), which we identified at ~110 kDa (Fig. 4A). Recently, proAKAP4 expression has been strongly correlated with total and progressive motility in stallion spermatozoa (Blommaert *et al.* 2018, Blommaert *et al.* 2019), and has, therefore, become the focus of many investigations into its use as a biomarker of ejaculate quality in dogs, bulls, boars, stallions and men (Delehedde *et al.* 2018, 2019, Le Couazer *et al.* 2019, Ruelle *et al.* 2019, Sergeant *et al.* 2019). Differences in AKAP4 abundance in the present study most likely stem from defective proAKAP4 expression. Herein, we confirmed previous findings that AKAP4 is significantly more abundant in high-quality spermatozoa isolated using density gradient centrifugation (Cui *et al.* 2016), and that AKAP4 expression is significantly correlated with motility parameters (Fanny *et al.* 2018, Blommaert *et al.* 2019) (Fig. 4D).

Although protein abundance was greater in all high-quality sperm fractions analyzed, we found that AKAP4 was a statistically stronger marker of sperm quality in samples collected from half of the stallions, while HK1 was statistically stronger in samples collected from the other half. Indeed, Fig. 2C, D and F highlight the extent of protein expression variability between stallions. These results indicate first; that the formation of low-quality sperm fractions in an ejaculate is not governed by one singular protein, and second; that some stallions may be more adversely affected by the under-expression of distinct proteins, giving rise to the formation of low-quality spermatozoa. It may be possible that molecular interactions can overcome particular defects that arise during protein synthesis. For instance, sperm tail proteins SPAG5 and ODF1 interact via leucine zipper motifs; deletion of which obliterates this interaction (Shao *et al.* 2001). However, SPAG5 contains an alternate leucine zipper motif that does not interact with ODF1, suggesting its ability to interact with other proteins to maintain functional capacity (Shao *et al.* 2001). In the case of results presented here, some stallions may possess the necessary machinery to surmount interruptions to the synthesis of a particular protein (e.g. AKAP4, but not HK1), thereby explaining the differences in statistical strength of sperm quality markers between individuals. It appears that the use of a single biomarker to predict stallion fertility may not be the most appropriate strategy. Rather, there is likely benefit in developing a robust,

multi-biomarker diagnostic platform, which accounts for inter-individual variability, and can simultaneously quantify a suite of biomarkers, increasing the predictive power of the assay.

The cytotoxic aldehyde 4-hydroxynonenal (4HNE) is a major end-product of lipid peroxidation, formed following the oxidation of polyunsaturated fatty acids (PUFA). Once produced, 4HNE covalently binds to the sidechains of amino acids, perturbing protein function (Esterbauer *et al.* 1991, Doorn & Petersen 2002, Dalleau *et al.* 2013) and leading to pronounced dysfunction in both male (Baker *et al.* 2015, Bromfield *et al.* 2015b, Moazamian *et al.* 2015) and female germlines (Lord *et al.* 2015, Mihalas *et al.* 2017). ProAKAP4 and AKAP4 have been identified as primary targets of 4HNE adduction in both round spermatids and mature spermatozoa (Baker *et al.* 2015, Nixon *et al.* 2019a). After treating cells with exogenous 4HNE, Baker *et al.* (2015) identified a number of AKAP4 peptides containing 4HNE carbonyl adducts, and reported a significant 11-fold increase in the number of modified residues present. These modified residues exist in close proximity to numerous important phosphorylation sites (Nixon *et al.* 2019a). In the present study, immunoprecipitation revealed significant adduction of AKAP4 by 4HNE (Fig. 5), while treatment with exogenous 4HNE resulted in a global reduction in phosphorylation profile (Fig. 6). Equine spermatozoa have a unique matrix of antioxidants that neutralize free radicals, but this ability is lost over time when antioxidant defences become overwhelmed (Gibb *et al.* 2016). The greater reduction in phosphorylation profile exhibited by the low-quality sperm fraction (Fig. 6C and D) may result from an inferior defense system exposing cells to detrimental oxidative attack more rapidly than higher quality cells.

Stallion spermatozoa are distinct in that their high dependence on oxidative phosphorylation means that markers of oxidative stress are positively correlated with sperm function (Gibb *et al.* 2014). As AKAPs are highly vulnerable to 4HNE adduction (Nixon *et al.* 2019a), in functional, high-quality cells, this may explain why equine sperm prove difficult to capacitate, and consequently why IVF in this species has rarely been achieved.

Immunoblotting revealed a significant loss or degradation of bands resolving at 64–70 kDa in non-capacitated, 4HNE treated samples (Fig. 6A and E). We suspect that these bands may represent a multiprotein complex involving heat shock-related 70 kDa protein 2 (HSPA2), arylsulfatase A (ARSA; 68 kDa) and the testis-associated serine proteases testisin (PRSS21; 70 kDa), among others. HSPA2 has been implicated as one of the initial targets of 4HNE-mediated modification (Bromfield *et al.* 2015b). HSPA2 functions in preventing the aggregation of misfolded proteins (Mayer & Bukau 2005), and therefore protection of other vital cell proteins from oxidative attack. In equine spermatozoa,

adduction of 4HNE to HSPA2 disrupts the HSPA2/ARSA/SPAM1 complex that participates in sperm-egg recognition; ultimately rendering the sperm incapable of fertilization (Bromfield *et al.* 2015a). ARSA – a protein vital for zona pellucida adhesion and binding (Carmona *et al.* 2002, Redgrove *et al.* 2012a,b, Bromfield *et al.* 2015a) – has been identified resolving at 68 kDa in capacitated boar sperm (Jiménez *et al.* 2006), and shares 90% sequence identity with equine ARSA. Testisin was recently characterized in stallion spermatozoa, and holds a functional relationship with HSPA2 (Swegen *et al.* 2019). Localized to both the sperm tail and the inner acrosomal membrane, testisin has been implicated as an integral member of the zona pellucida-binding complex (Netzel-Arnett *et al.* 2009, Swegen *et al.* 2019). The significant degradation of bands resolving at 64–70 kDa in the present study confirms previous findings that, even in low concentrations, cytotoxic aldehydes can have a significant effect on overall mammalian cell function (Bromfield *et al.* 2015a,b, Hall *et al.* 2017).

Driven by the limitations imposed by the Thoroughbred breeding and racing industries, there is increasing pressure to produce foals close to the commencement of the breeding season. Decreases in stallion fertility generate a host of economic and welfare implications concerning both mares and stallions. Increasing our knowledge of the molecular factors that govern stallion fertility, will pave the way toward identifying putative biomarkers of sperm function. Such breakthroughs would facilitate the close monitoring of the molecular changes – predictive of future fertility outcomes – via the regular collection of post-coital dismount semen samples. Sperm populations collected from dismount samples are highly representative of whole ejaculate quality (Gibb *et al.* 2014). The results of such analyses will enable the timely adjustment of management strategies, well before mares begin to return negative pregnancy diagnoses.

In conclusion, we identified several proteins whose expression is distinctly different between high- and low-quality stallion spermatozoa isolated from the same ejaculate, and established their functional significance by highlighting significant correlations present between protein abundance and motility. The research findings presented herein will go a long way toward the development of a robust, multi-biomarker diagnostic platform, capable of accurately predicting sperm function – the development of which could potentially improve overall horse welfare and the economic conditions surrounding Thoroughbred ownership. Further research is needed to investigate the usefulness of AKAP4 and HK1 for predicting times of high and low fertility in the field.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0284>.

Declaration of interest

Maryse Delehedde and Nicolas Sergeant are both co-founders of the SPQI company (Lille, France) that commercialise the AKAP4 antibody used in this study. Robert Aitken is on the editorial board of *Reproduction*. Robert Aitken was not involved in the review or editorial process for this paper, on which he is listed as an author.

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Author contribution statement

Z G, A S and R J A initiated and designed the study with input from R A G, M B, B N, M D and N S. R A G performed experiments and data collection. UHPLC-MS/MS was facilitated by F J P and performed by A S R and F E M-C. Data analysis was performed by R A G with assistance from M B and D A S-B; facilitated by B N. R A G drafted the original manuscript which was sent to all authors for correction.

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