Journal of Proteomics 157 (2017) 1–9

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Proteomic characterization of seminal plasma from alternative reproductive tactics of Chinook salmon (*Oncorhynchus tswatchysha*)

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ARTICLE INFO

Article history:
Received 8 October 2016
Received in revised form 24 January 2017
Accepted 28 January 2017
Available online 2 February 2017

Keywords:
Seminal plasma
Fertility
Spermatogenesis
Salmon
Proteomics
Ion mobility
Sperm competition

ABSTRACT

Chinook salmon (*Oncorhynchus tshawytscha*) are external fertilizers that display sneak-guard alternative reproductive tactics. The larger hooknose males dominate mating positions, while the smaller jack males utilize sneak tactics to achieve fertilization. Although poorly understood, previous studies have suggested that differences in spermatozoa quality may play a critical role in sperm competition. Considering that the seminal plasma strongly regulates spermatozoa quality and other processes critical for fertilization success, we employed label free quantitative mass spectrometry utilizing ion mobility separation coupled to cross-species bioinformatics to examine the seminal plasma proteome of Chinook salmon. A total of 345 proteins were identified in all biological replicates analyzed, including many established seminal plasma proteins that may serve as future biomarkers for Chinook salmon fertility and sperm competition. Moreover, we elucidated statistically significant protein abundance differences between hooknose and jack male tactics. Proteins involved in membrane remodeling, proteolysis, hormonal transport, redox regulation, immunomodulation, and ATP metabolism were among the proteins reproducibly identified at different levels and represent putative factors influencing sperm competition between jack and hooknose males. This study represents the largest seminal plasma proteome from teleost fish and the first reported for Chinook salmon.

Significance: Chinook salmon (*Oncorhynchus tshawytscha*) males represent an example of male alternative reproductive tactics where diverse reproductive strategies are thought to increase sexual selection. While seminal plasma has been shown to play an important regulatory role in sperm competition in many species, little is known about the protein composition of the seminal plasma of salmon. Therefore, seminal plasma isolated from the two alternative reproductive tactics of Chinook salmon (small sneaky jacks and large dominant hooknoses) were analyzed by label free quantitative mass spectrometry employing data independent acquisition and ion mobility separation. This yielded the largest proteome data set of the seminal plasma from salmon and the first to examine protein abundance differences between male alternative reproductive tactics. The quantitative proteomic data provides insight into possible unique mechanistic aspects of Chinook salmon alternative reproductive tactics utilized for sperm competition and fertilization success.

1. Introduction

In external fertilizing fishes (including salmonids), the efferent duct produces seminal plasma, providing an optimal ionic environment for maintaining the viability of spermatozoa after their release from the testes into the sperm duct and once released into the spawning environment [1]. In addition to maintaining the quality of spermatozoa inside the reproductive tract or in the spawning environment, recent research has shown that seminal plasma also plays a role in sperm competition [2]. Competition occurs when spermatozoa from multiple males contend over the chance to fertilize a female's eggs [2] and is especially prevalent in fish species in which male alternative reproductive tactics are present [3]. The most common alternative reproductive tactic system seen across fish taxa is the sneak-guard dichotomy in males [4]. Sneaker males usually have small body size and use covert techniques to sneak into mating events between guard males and females to obtain reproductive success. In contrast, the guard males are typically large in body size and have more pronounced secondary sexual characteristics to aid in asserting dominance, including fighting off other males while protecting and monopolizing females. The prevailing theory in the

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http://dx.doi.org/10.1016/j.jprot.2017.01.019
1874-3919/

field predicts that sneaker males invest more into spermatogenesis instead of body size, resulting in higher quality sperm, while the guard males invest more into body size and secondary sexual characteristics in order to defend females or resources important to females [5]. For example, in Atlantic salmon (*Salmo salar*), the precocious parr (sneaker male), relative to body size, has larger testes, ejaculate volume, number of spermatozoa cells, higher spermatozoa motility, and live longer compared to the anadromous (guard) males [6,7].

Chinook salmon (*Oncorhynchus tshawytscha*) also exhibit the sneak-guard alternative reproductive tactic in males, where the large, dominant hooknose (guards) have priority in mating positions with females, while the small, precocious jack males (sneakers) adopt the sneaking tactic [8,9]. Previous work has shown that jacks have relatively larger testes and their spermatozoa swim faster in river water compared to hooknose [10], supporting the theory that sneaker males (jack) possess higher sperm quality in general [11]. However, differences in testes size may also result in seminal plasma variation between jack and hooknose males that could impact fertilization success.

Most of the studies to date that examine sperm competition dynamics in fishes have focused on either looking at differences in spermatozoa number or spermatozoa quality [12]. However, spermatozoa only make up a portion of the ejaculate and other components, such as seminal plasma (fluid) can have effects on the outcome of sperm competition [13,14]. For example, Locatello and colleagues [15] demonstrated that for the grass goby (*Zosterisessor ophiocephalus*) the seminal plasma from the sneaker tactic can attenuate the performance of the rival guards' spermatozoa. Furthermore, spermatozoa from sneaker males were shown to achieve higher velocity and fertilization success by utilizing the guard male's seminal plasma [15].

Recent advances in quantitative proteomics have given rise to label-free techniques for performing comparative analysis of protein levels between different samples [16]. Label-free techniques typically compare normalized precursor ion intensities across multiple biological and technical replicates to detect differential protein levels [16]. Furthermore, label-free approaches combined with data independent acquisition modes and orthogonal separation techniques, such as ion mobility separation, greatly enhances reproducibility, sensitivity, and sequence coverage compared to data dependent spectral counting techniques [17,18]. Thus, the promise of proteomics has made it a powerful approach to analyze protein dynamics in the male gametes of aquatic animals [19–22]. However, despite the increasing evidence that seminal plasma is an important factor influencing spermatozoa quality and fertilization competition, little information is available on its protein composition in Chinook salmon. In this study, we aimed to advance the understanding of the Chinook salmon seminal plasma proteome, and determine functional differences between alternative reproductive tactics of Chinook salmon from a wild spawning population using label-free quantitative proteomics.

2. Materials and methods

2.1. Collection of Chinook male tactics

Sexually mature Chinook salmon were collected during two spawning sessions (2–6 October, 2013 and 4–6 October, 2014) using backpack electrofishing from a winter run in the Credit River (Mississauga, Ontario, Canada, N 43°35′, W 79°42′), which flows into Lake Ontario (that has been stocked for over 40 years) [11,23–25]. We considered males <675 mm in fork length to be jacks based on length-age relationships derived from otolith assessment for our population. All males >675 mm were considered hooknose males and documented as age 1+ (675–750 mm), age 2+ (750–850 mm) or age 3+ (900–950 mm) [8,10–11] (Ontario Ministry of Natural Resources, unpublished data). In addition, all fish <675 mm in fork length in our population were morphologically characteristic of jacks (e.g. coloration similar to that of females).

Chinook salmon were located upstream in turbid water ranging from 2 to 4 ft in depth. Water temperature at the time of collection was ~11 °C. The Ontario Ministry of Natural Resource and Forestry technicians (License number 1081183) humanely sacrificed the fish and obtained milt (fluid containing spermatozoa and seminal plasma) samples by applying pressure on the abdomen of each fish. The initial male ejaculate was discarded in a standardized manner and the external urogenital pore was wiped dry to avoid contamination from water, urine, feces, and blood. Milt samples were collected into 532 mL clear Whirl-Pak sample cooling bags (Nasco, Newmarket, ON, Canada) and were stored on ice in coolers for approximately 4 h during transport back to the laboratory for immediate processing. All research followed the University of Windsor and Canadian Council of Animal Care guidelines (AUPP 14–25).

2.2. In-solution trypsin digest of seminal plasma samples

The milt samples from five biological replicates per tactic (5 jack males and 5 hooknose males) were subjected to centrifugation at (300 $\times g$) for 10 min to separate seminal plasma and spermatozoa cells. Seminal plasma protein concentration was determined by Bradford assay. Equal amounts of total protein from each biological specimen was precipitated overnight using a chilled 4:1 acetone solution at −20 °C. Protein pellets were washed three times with acetone and re-suspended in solubilization buffer containing 0.1% RapiGest SF surfactant (Waters) in 50 mM ammonium bicarbonate (Fisher Scientific). Protein samples were reduced and alkylated with 10 mM dithiothreitol (Fisher Scientific) and 55 mM iodoacetamide (Sigma), subjected to in-solution trypsin digestion (Promega) at a 1:50 (enzyme:protein) ratio and incubated overnight at 37 °C. Trifluoroacetic acid (Pierce) was added to each sample the next day at a final concentration of 0.5% to quench enzyme activity and hydrolyze the surfactant according to manufacturer's protocol. All samples were de-salted using OASIS HLB extraction columns (Waters). Peptides were eluted in 65% acetonitrile (Burdick & Jackson) and concentrated by vacuum centrifugation. The peptides were then re-suspended in 0.1% formic acid, 3% acetonitrile and supplemented with Hi3 internal peptide standards (Waters) at a final concentration of 12.5 fmol/μL for quantitation.

2.3. Liquid chromatography-mass spectrometry

Generated tryptic peptides were separated on a 1.7 μm BEH130 100 μm × 100 mm reverse phase column (Waters) at a flow rate of 0.3 μL/min using a nanoAcquity UPLC system (Waters). Mobile phase A consisted of 0.1% formic acid in H_2O and mobile phase B consisted of acetonitrile with 0.1% formic acid. From a 5 μL sample loop, 4 μL was injected onto a 180 μm × 20 mm C18 pre-column trap for 3 min at 5.0 μL/min. Peptides were then separated on a 120 min gradient (3–25% B for 70 min, 25–50% B for 20 min, 50–85% B for 9 min, 85–3% for 2 min, 3% B for 20 min) and electrosprayed into a SYNAPT G2-*Si* mass spectrometer (Waters). The mass spectrometer was operated in data independent acquisition mode employing ion mobility separation ($HDMS^E$) alternating between low energy (4 eV) and high energy (20–45 eV) scans in positive resolution mode scanning from 50 to 2000 *m/z* with a scan rate of 0.6 s. [Glu1]-fibrinopeptide B (50 fmol/μL) was used as the lock mass external calibrant. Cone voltage was set to 30 V, and the capillary voltage was set at 3 kV. MassLynx (version 4.1) was used to collect the data. The raw data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005533.

Processing was performed using Progenesis QI (Nonlinear Dynamics) for peptide identification analysis and a teleost subset of the NCBI database (the teleost database consisted of 1,000,974 proteins and was downloaded on October 10, 2014). The following processing parameters were used: low energy noise reduction thresholds-135, high-energy threshold-30, and intensity threshold-750, with data lock

mass corrected post acquisition. Search parameters included a maximum protein mass of 250 kDa, a minimum of two matched peptides per protein, a minimum of seven fragment ion matches per protein, a minimum of three fragment ion matches per peptide, a maximum of two missed cleavages (trypsin). The false-discovery rate was estimated to be below 1% using a decoy database. Variable modifications for carbamidomethylated cysteine and oxidation of methionine were specified. Three technical replicates for each of the five biological replicates were analyzed per tactic (30 total measurements). Quantitation was determined using the three most abundant peptides per protein (including the internal standard) and normalized using the Progenesis QI software. The normalization processing utilized a reference chromatography run and spiked Hi3 internal standard peptides (50 fmol on column) to convert raw abundance values to absolute measurements (fmol on column) [26].

2.4. Statistical analysis and bioinformatics

Differences in protein abundance was determined by excluding proteins identified with less than three matched peptide products and with an ANOVA score (p-value) > 0.05. A Student's t-test was performed comparing the average amount of protein (fmol) from the five biological replicates of hooknose and jack males. Protein values for each biological replicate were obtained by calculating the average protein abundance from each corresponding technical replicate (in triplicate). The p-values obtained were corrected for false-discovery by applying the Benjamini-Hochberg method of analysis to produce a corrected t-test value (q value) [27]. All q-values <0.05 were considered significant and protein abundance ratios >1.5 were considered significant. A Volcano-plot was constructed by using the "ggplot2" package on "R" statistical software by plotting the $\log_2$fold change (x-axis) in relation to the $-\mathrm{Log}_{10}$ p-value (y-axis) [28,29]. Gene ontology was assigned to the identified seminal plasma proteins by sequence alignment using BLAST+ (Version 2.2.30) against the Human Uniprot/SwissProt database. The protein accession numbers obtained from the BLAST+ analysis were uploaded onto UniprotKB to map gene ontology.

2.5. Immunoblot analysis

For immunoblots assays, 10 μg of seminal plasma protein from selected biological specimens were loaded onto 12% SDS/PAGE gels. Alternatively, 25 μg of pooled seminal plasma from jack and hooknose samples was subjected to exosome isolation using the Total Exosome Isolation Kit (Invitrogen) according to the manufacturer's instructions. Following SDS/PAGE analysis, the samples were electrotransferred onto PVDF membranes and incubated for 1 h at room temperature with a cross reacting rabbit lactate dehydrogenase (LDH) antibody raised against LDH from *Toxoplasma gondi* (kind gift from Dr. Sirinart Ananvoranich) at a 1:5000 dilution in 2.5% dry milk (v/v)/TBST (20 mM Tris Base, 140 mM NaCl, 0.1% Tween 20, pH = 7.6) or a cross reacting rabbit pan 14-3-3 antibody raised against human 14-3-3 (Santa Cruz Biolabs) at a 1:5000 dilution in 2.5% dry milk/TBST and anti-rabbit HRP (Biorad) at a 1:5000 dilution made with 2.5% milk/TBST at room temperature for 45 min. Proteins were visualized using Super Signal West Femto Reagent (Thermo Scientific).

3. Results and discussion

3.1. Identification of hooknose and jack seminal plasma proteins by data independent ion mobility mass spectrometry

To better characterize the seminal plasma protein profiles of hooknose and jack Chinook salmon males, a cross-species proteomics and bioinformatics workflow was utilized (Fig. 1). Ten sexually mature biological specimens (five jack males and five hooknose males) were collected from the field and the milt was isolated. It is important to note that jack males sexually mature one year earlier than hooknose males [8–11]. Moreover, during fertilization competition, a younger jack male will generally be competing with an older hooknose male (younger in terms of age, but equal in terms of sexual maturity). Thus, although we cannot rule out contributions of age to differences in the seminal plasma proteome, it was imperative to compare the seminal plasma proteome at the stage of equal sexual maturity for the purpose of investigating protein factors contributing to fertilization competition.

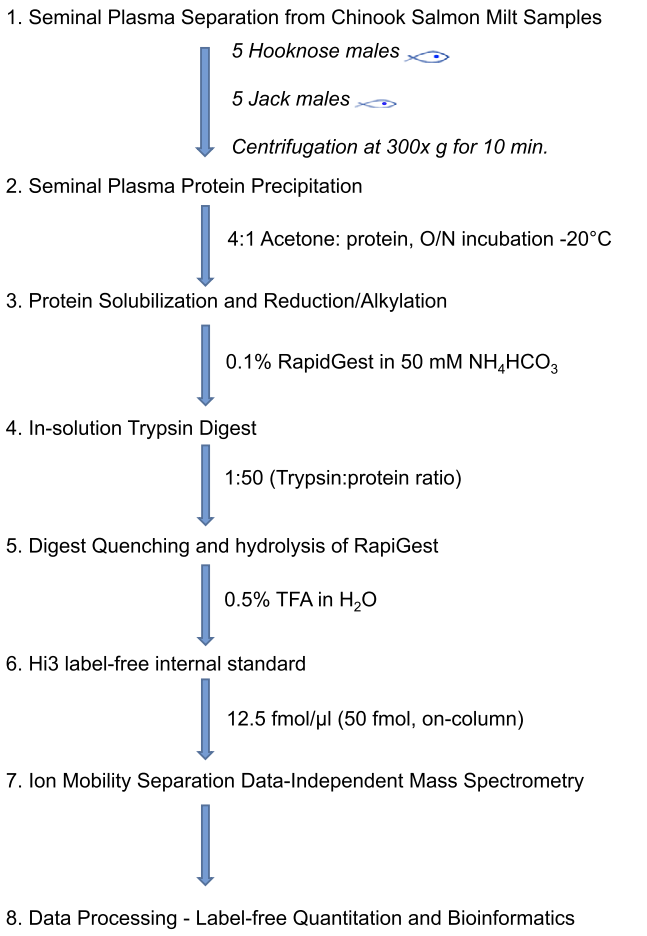

Fig. 1. Schematic workflow of seminal plasma sample preparation and proteomic analysis. Seminal plasma was extracted from milt by centrifugation, and proteins were prepared for mass spectrometry analysis using RapiGest solubilization and in-solution trypsin digestion (Steps 1–5). Label-free internal standards (Hi3) were added to each sample for absolute quantitation (Step 6). Samples were analyzed by UPLC ion-mobility data-independent mass spectrometry (Step 7) and the data was processed using Progenesis-QI (Step 8). Statistical analysis was performed to determine significant differences in protein abundance (Step 8).

Seminal plasma was isolated and processed for mass spectrometry analysis (see "Materials and Methods" for details). Furthermore, synthetically prepared internal standards based on peptide sequences found in the *E. coli* protein ClpB (Hi3) were added to all biological replicates for absolute quantitative analysis. Protein identification was accomplished using data independent tandem mass spectrometry (LC-MS^E). To increase the number of unique proteins identified and increase protein sequence coverage, ion mobility separation of the peptides was performed using the traveling wave function of the SYNAPT G2-*Si* system located between the quadrupole and time of flight cell. Preliminary scout runs determined that incorporating ion mobility separation in the workflow increased the number of proteins identified by ~40% (data not shown) demonstrating the significant improvement in proteome coverage when ion mobility separation is employed as reported by other proteomic studies employing ion mobility separation [18,30]. In total, three

technical replicates were analyzed for each of the ten biological replicates (five from hooknose and five from jack males).

The Chinook salmon genome is poorly annotated [31] with available protein databases having <900 putative protein sequences for proteomic analysis. With the knowledge that conservation between closely related organisms increases the probability of identification in poorly characterized species [32–34], the teleost (bony fish) database was utilized for protein identification. Whole sequence protein alignments resolved conflicts and filtered out protein redundancy, while BLAST analysis assigned protein identification to predicted proteins (Table S1). After eliminating proteins identified by one or two peptides and proteins not detected in all thirty technical replicates, our analysis yielded 345 total proteins found in Chinook salmon seminal plasma (Tables S1 and S2). Furthermore, the addition of Hi3 internal standards to all of the biological replicates allowed for label-free quantitation of identified proteins using the Progenesis QI software package [16,35]. Analysis of relative abundance and calculated on-column peptide levels (fmol) revealed a dynamic range that spanned 4 orders of magnitude, although the majority of proteins were within a dynamic range of 3 orders of magnitude (0.1–500 fmol on column) (Fig. 2). At the high end of the dynamic range were well-characterized seminal plasma proteins such as transferrin, albumin-2, and alkaline phosphatase while at the low end of the dynamic range were known seminal plasma proteins DJ-1 and ceruloplasmin [36]. Collectively, LC-MS^E using ion mobility separation yielded the first described proteomic data set for Chinook salmon seminal plasma, and to our knowledge, the largest seminal plasma proteome reported for teleost fish.

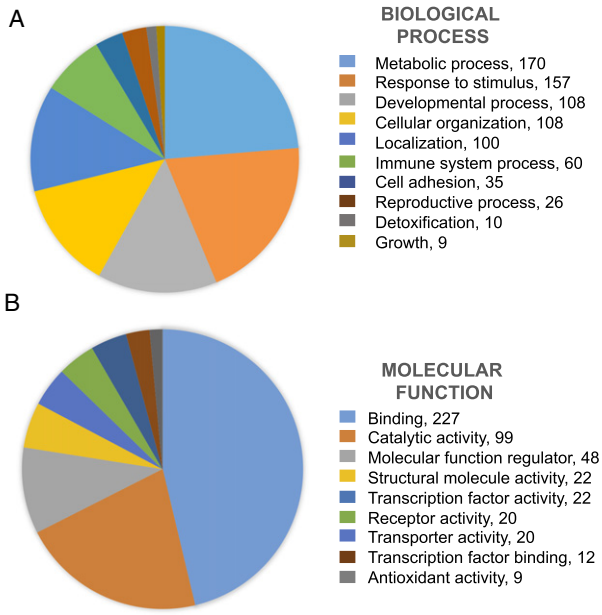

Fig. 3. Gene ontology of seminal plasma proteins identified in Chinook salmon (*Oncorhynchus tswatchysha*) jack and hooknose seminal plasma. A) Gene ontology mapped for seminal plasma proteins in relation to biological process. B) Gene ontology mapped for seminal plasma proteins in relation to molecular function. Gene ontology terms are shown in adjacent legend with corresponding number of matching proteins.

3.2. Seminal plasma proteins identified in hooknose and jack Chinook salmon males

Gene Ontology (GO) bioinformatic analysis provides an overview of the dominant functional traits of isolated proteomes. Of the 345 proteins identified that were common in the seminal plasma fluid of both Chinook male species, 269 (78%) were successfully mapped to a GO (Fig. 3). For the biological process category, metabolism and cellular regulation/signaling are most represented in the proteome (Fig. 3A). With regards to molecular function, the predominant gene function activities represented in the Chinook seminal fluid are binding activity and catalytic activity (Fig. 3B). A similar GO profile was observed in the closely related rainbow trout seminal plasma [37] and carp seminal plasma proteome studies [38], providing a level of validation for the identified proteins. GO analysis of our findings further supports the notion that the principle components of teleost seminal fluid consists of immunomodulators and redox regulators for spermatozoa survival, metabolic factors for energy requirements during fertilization, and proteolytic enzymes/inhibitors for temporal regulation of these processes (Figs. 4, Table S1).

In addition to a common gene ontology profile, most of the specific proteins identified in previous fish seminal plasma proteomic studies from rainbow trout [37] and carp [38] were also identified in our dataset (Table S1). For instance, several members of the complement

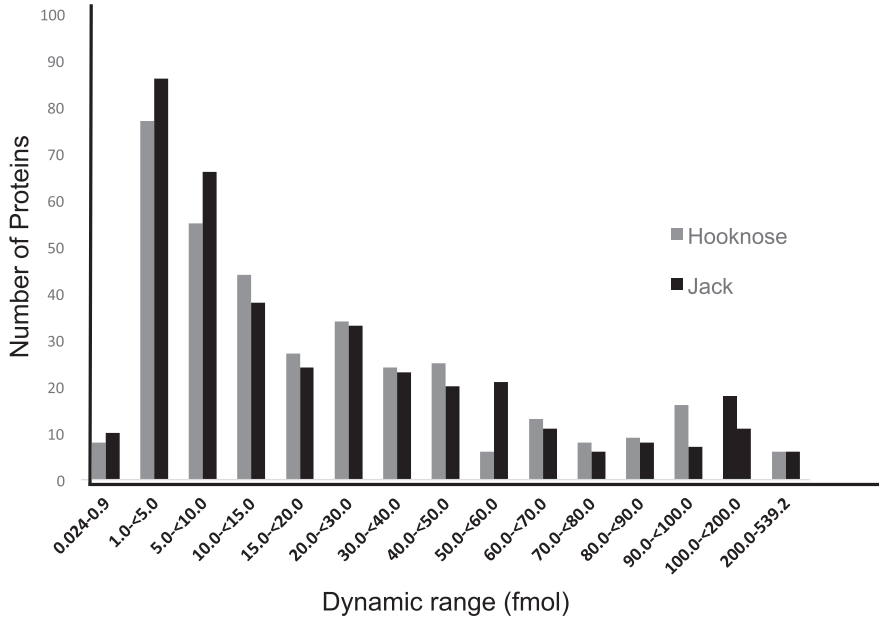

Fig. 2. The dynamic range of on-column protein abundance identified from Hooknose and Jack seminal plasma. The distribution of proteins (y-axis) within a range of on-column protein abundances in fmol (x-axis) for both hooknose and jack males. Grey bars represent hooknose proteins and black bars represent jack proteins.

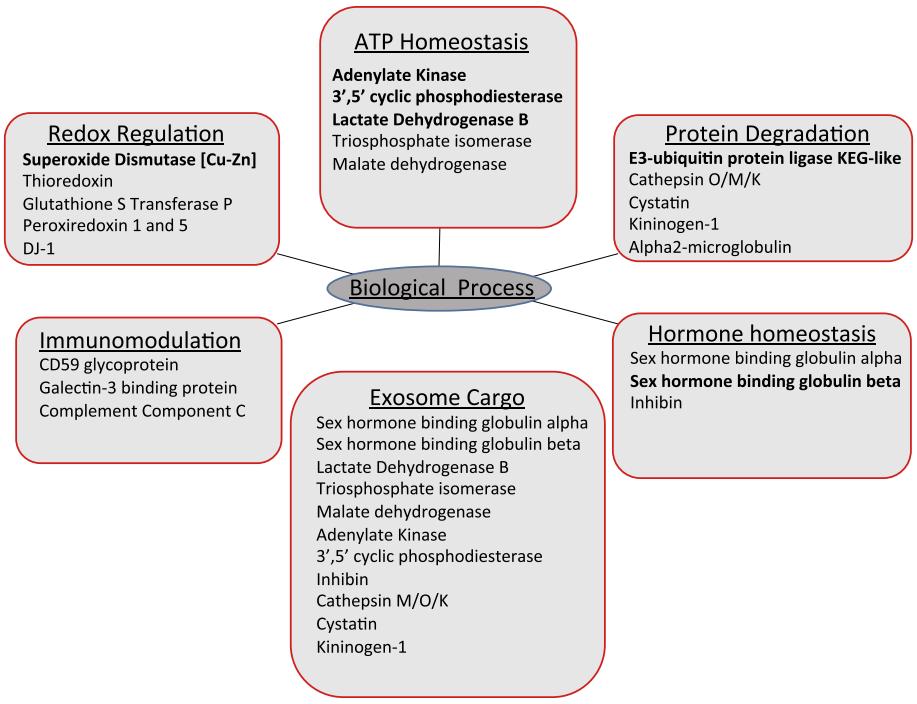

Fig. 4. Representative seminal plasma proteins identified in Chinook salmon. Identified proteins of interest were categorized under biological processes known to be critical for seminal plasma biology. Highlighted in bold are proteins whose protein abundances were observed to be statistically significant (Fig. 7).

component family (C3, C3–3, C3–4, C6, C8α, C8β and C9) were identified. These proteins have a well-characterized role in the innate immune response [39] and may possibly be associated with sperm competition [39,40]. Apolipoproteins (AI, AII, AIV, E and F) involved in lipid transport, metabolism, as well as the immune response [41] were also identified in our study, consistent with past fish seminal plasma proteomic studies [37,38].

3.3. Potential Chinook salmon biomarkers relevant to sperm fitness

In addition to the complement of proteins reported in previous teleost studies, the present proteomic work also identified novel teleost seminal plasma proteins, including certain proteins that may be suited to serve as biomarkers related to sperm quality (Table S1). One example is the identification of inhibin, which is novel for fish seminal fluid, but has been identified in human seminal plasma [36]. Inhibin is an endocrine factor that appears to regulate hormone homeostasis for female and male reproductive organs, and has been proposed to be a biomarker of spermatogenesis [42,43]. Another example is the protease inhibitor alpha-2-microglobulin (α2M) [44,45], discovered in the seminal plasma of both tactics (Fig. 4, and Table S1). α2M has been proposed to serve as a biomarker for human sperm quality as increasing concentrations of α2M have been correlated to progressive forward motility [46]. α2M has a broad specificity of protease targets and thus affects a diverse range of cellular processes [47]. Interestingly, the aforementioned protein inhibin, is a known binding partner of α2M [48]. The levels and activities of inhibin and α2M in Chinook salmon warrant further exploration to determine their suitability as a biomarker for spermatozoa fitness.

Orthologues of many of the proteins identified in our study have been associated with extracellular vesicles called exosomes (Fig. 4). Most cell types secrete exosomes, and are found in a variety of bodily fluids including seminal plasma [49]. Exosomes are emerging as powerful biomarkers for use in diagnostic and prognostic analysis of a variety of human disorders [49–54]. With regards to seminal plasma, exosomes have been shown to play an immunosuppressive role in protecting spermatozoa from the male immune system [53,54]. Furthermore, seminal exosomes are thought to deliver protein cargo and other biomolecules to the spermatozoa to facilitate fertilization [55–57]. In our data set, both tactics possessed proteins implicated in regulating the export of exosomes, such as rab11 family interacting protein [49,58], and a variety of proteins previously described as exosome cargo (Fig. 4). Most notably, immunosuppressive proteins CD59, and galectin-3 binding protein were identified and have been shown in other species to reside in exosomes [59–61]. The levels of these and other exosome cargo may be useful indicators of sperm fertilization fitness for both Chinook male tactics.

3.4. Quantitative protein differences in the seminal plasma of individual tactics of Chinook salmon

A major objective of our study was to elucidate differences in protein levels between hooknose and jack seminal plasma as a means to better understand tactic specific reproductive fitness. Proteins identified in all technical and biological replicates were considered for label free quantitative analysis and statistically significant protein differences were determined (see "Materials and Methods" for details). A volcano plot displaying normalized $\log_2$ protein abundance ratio against statistical significance measurements ($-\log_{10}$ q value) is shown in Fig. 5. Overall we observed 29 proteins with statistically significant protein level differences and calculated abundance ratios of at least 1.5 (Fig. 5 and Table S1) [62,63]. GO analysis of these altered proteins revealed a similar GO distribution between the tactics and did not deviate significantly from the predominant molecular functions and biological processes of the proteins that are common between hooknose and jack males (Fig. 6). This suggests that the seminal plasmas of hooknose and jack males generally are functionally equivalent in terms of protein classifications. However, attention to the specific protein differences suggests mechanistic and functional diversity between the tactics, including proteins regulating hormonal transport, ATP metabolism, redox regulation, and

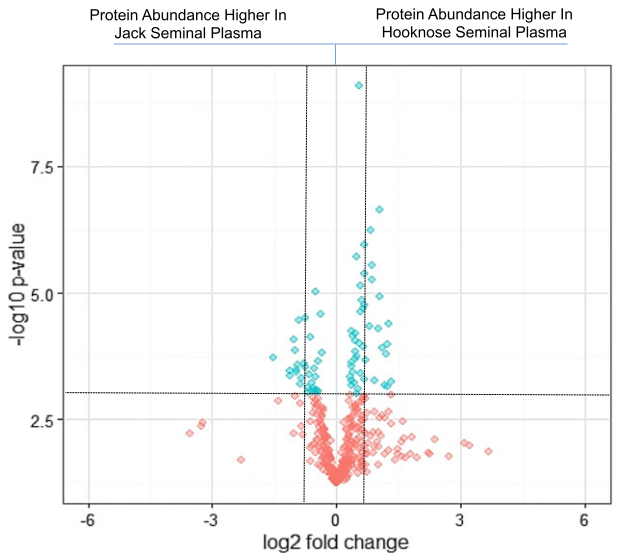

Fig. 5. Volcano plot depicting differentially abundant seminal plasma proteins between hooknose and jack males. The volcano plot shows $-\log_{10}$ corrected *t*-test values (q-value) plotted against $\log_2$ fold change (x-axis). Data points above horizontal dashed line (q values below 0.05) and to the left and right of the vertical dashed lines (abundance ratios above 1.5) represent proteins with statistically significant abundance differences (highlighted in blue).

proteolysis (Fig. 7, and Table 1). For example, sex hormone binding globulin (SHBG) was found at significantly higher levels in jack males than in hooknose males (Fig. 7, and Table 1). SHBG is a well-characterized steroid hormone transporter that regulates androgen/estrogen availability to target tissues [64]. Furthermore, the steroid hormone targets for SHBG are known modulators for spermatogenesis, spermatozoa maturation, and motility acting through non-genomic mechanisms [65,66]. Therefore, higher amounts of SHBG in jack males suggest a greater capacity for hormone-mediated effects in the seminal plasma of this tactic.

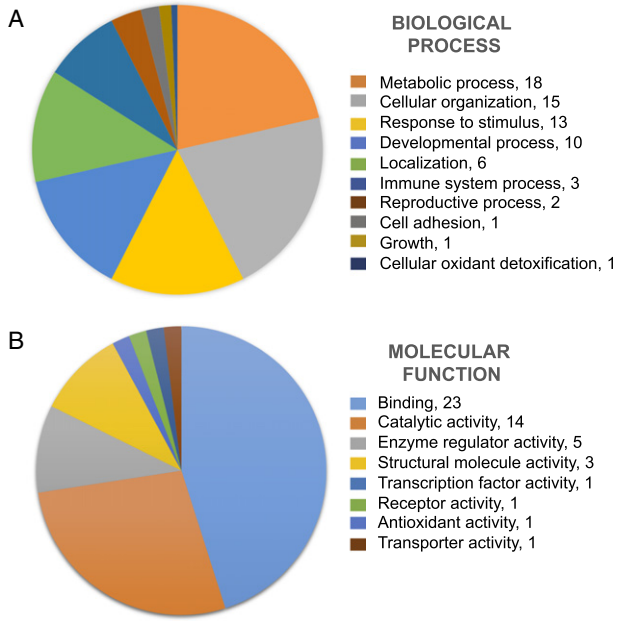

Fig. 6. Gene Ontology for seminal plasma proteins differentially abundant in Chinook salmon (*Oncorhynchus tswatchysha*) jack and hooknose seminal plasma. Gene ontology for proteins whose levels were determined to be statistically significant in jack and hooknose seminal plasma in relation to molecular function (A) or biological process (B).

Another intriguing finding was the observed increase in the enzyme cGMP-inhibited 3′,5′-cyclic phosphodiesterase (PDE) in the jack males. PDE catalyzes the hydrolysis of cAMP, a potent second messenger that regulates numerous spermatozoa related activities such as motility and capacitation [67,68]. Extracellular PDE is widely reported in literature and has also been characterized in the seminal plasma of numerous organisms [69,70]. The difference in PDE levels between the tactics insinuates that hooknose males, by virtue of having lower levels of PDE, may exhibit more prominent cAMP signaling than jack males.

In addition to factors affecting cAMP activities, levels of enzymes regulating additional metabolic compounds were found to be different between hooknose and jack males. Interestingly, the nucleotide converting enzyme adenylate kinase (AK) was detected at a two-fold higher level in hooknose males (Fig. 7, and Table 1). AK catalyzes the reversible reaction of ATP + AMP = 2ADP and thus represents an important mediator of bioenergetic sensing [71]. AK is detected in extracellular fluids, including seminal plasma [72,73], and has been reported to mediate ATP shuttling to ATPases residing in the spermatozoon tail, facilitating motility [74].

One of the most prominent protein differences was a two-fold abundance ratio increase in lactate dehydrogenase (LDH) in jack males (Fig. 7A, and Table 1). Interestingly, we were able to validate the quantitative proteomic data by immunoblot analysis using a cross reacting LDH antibody (Fig. 7B). Overall, we observed higher amounts of LDH in jack males compared to hooknose males, although one hooknose individual (H113) displayed elevated LDH levels by immunoblot analysis and quantitative proteomics. Furthermore, as LDH has been characterized to be a component in seminal plasma exosomes [56], we isolated exosomes from pooled seminal plasma and observed higher levels of LDH from jack males compared to hooknose males (Fig. 7C). As a control we also probed for 14-3-3 using a pan 14-3-3 antibody. This protein is a known exosome protein [51–52] and was found to be equally abundant in our proteomic analysis as well as in our isolated exosome samples (Fig. 7C). It has been proposed in several species that spermatozoa can utilize lactate found in the seminal plasma as an energy source, whereby LDH in the seminal plasma converts lactate to pyruvate and NADH that is subsequently transported into the sperm's mitochondria for oxidative phosphorylation and ATP production [75]. This metabolic system is critical for meeting the energy demands of motile spermatozoa and thus is a contributing factor for producing high quality sperm [76]. Furthermore, this finding is consistent with past studies that have predicted jack males to participate in sperm competition by the so-called “loaded raffle” mechanism, whereby sneaker males (jacks) possess higher quality spermatozoa in order to compete for reproductive success [10]. Thus, further detailed mechanistic studies will be important to examine if jack males utilize higher levels of LDH in their seminal plasma to provide their spermatozoa a metabolic advantage over hooknose spermatozoa.

A notable class of proteins identified in the Chinook salmon seminal plasma of both tactics was those that participate in oxidative stress resistance such as thioredoxin, peroxiredoxin, glutathione transferase, and DJ-1 (Table S1). Oxidative insults from reactive oxygen species (ROS) during the shift from hypoxic to normoxic conditions during external fertilization can be detrimental to spermatozoa cells [77]. Thus, up-regulation of antioxidant factors enhances sperm viability by increasing survival towards oxygen radicals. ROS have also been shown to have a degradative effect on spermatozoa performance such as motility, but help enhance immune function and increase capacitation in humans [78]. Strikingly, our study found superoxide dismutase (Zn/Cu SOD) levels to be greater than two-fold higher in hooknose males (Fig. 7A, and Table 1). SOD levels in seminal plasma have been shown to correlate to ROS tolerance, protecting spermatozoa from oxidative damage [79]. However, recently reports have demonstrated that SOD activity inhibits spermatozoa capacitation by eliminating localized superoxide

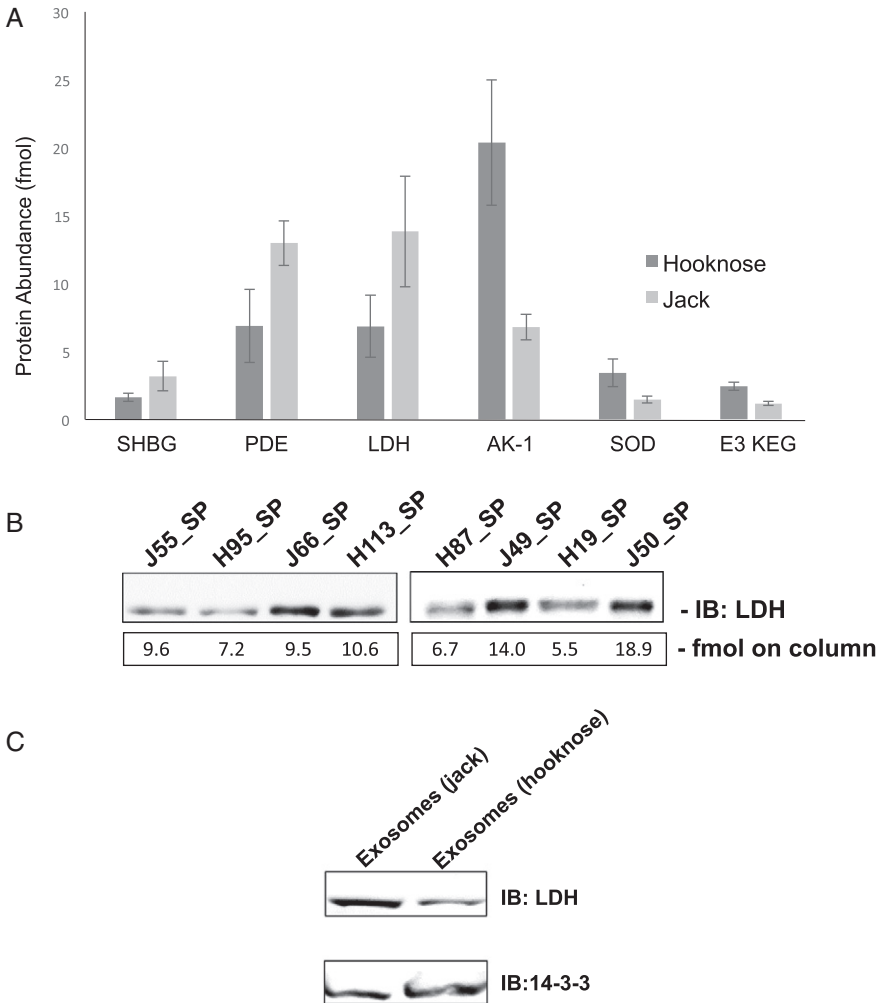

Fig. 7. Absolute quantitation of selected proteins from jack and hooknose seminal plasma. A) Protein abundances are shown as mean fmol (on-column) ± standard deviation (see Materials and Methods for details). All protein comparisons shown were statistically significant, $p < 0.001$. Individual statistical values are displayed in Table 1. Proteins shown are the following: Sex hormone binding globulin (SHBG), 3′,5′-cyclic phosphodiesterase (PDE), lactate dehydrogenase (LDH), adenylate kinase 1-1 (AK-1), superoxide dismutase (SOD), and E3 ubiquitin ligase KEG-like (E3 KEG-like). B) Seminal plasma (10 μg) from individual biological specimens was analyzed by LDH immunoblotting using a cross reacting rabbit LDH antibody. The corresponding on-column fmol amount determined by mass spectrometry for the individual biological specimens is shown. C) Isolated exosomes from 25 μg of pooled jack and hooknose seminal plasma were subjected to immunoblot analysis using anti-LDH and anti-14-3-3 antibodies.

Table 1
Functional classification of differentially abundant proteins between hooknose and jack males. Proteins whose abundance ratios >1.5 and had *p*-values and FDR adjusted q-values <0.05 were considered significant. Proteins are organized by abundance ratios and which tactic contained the higher abundance; hooknose (upper panel), jack (lower panel).

Tactic	*p*-Value	q-Value	% CV	Fold change	Protein description	Protein function
Hook	1.44E-09	0.006025	1.74	2.4	Superoxide dismutase_P03946.2	Redox homeostasis
Hook	2.12E-06	0.041019	2.80	2.3	Adenylate kinase 1_ACH70899.1	ATP shuttling
Hook	2.38E-08	0.019191	4.79	2.2	Spidroin-1-like_XP_014065990.1	Extracellular matrix organization
Hook	1.23E-09	0.015444	2.12	2.1	RNA-binding motif protein_XP_014067707.1	Alternative splicing
Hook	1.67E-15	0.000319	3.76	2.1	E3 ubiquitin ligase KEG_XP_014062379.1	Protein ubiquitination/degradation
Hook	1.90E-05	0.034955	2.08	1.9	Creatine kinase B-type_EMP32891.1	ATP homeostasis
Hook	3.52E-11	0.001165	2.86	1.8	Apolipoprotein C-I_NP_001134834.1	Membrane stabilization
Hook	2.37E-11	0.001747	1.95	1.8	Lumican precursor_ACI33424.1	Extracellular matrix organization
Hook	9.36E-07	0.007213	1.06	1.7	Semaphorin-4F-like_XP_014065278.1	Regulation of cell migration
Hook	7.40E-10	0.000674	4.38	1.6	Transcription factor Sox-19a_XP_014051352.1	Transcription factor
Hook	1.75E-10	0.001586	1.65	1.6	Creatine kinase_P24722.1	ATP homeostasis
Hook	2.83E-06	0.026153	2.84	1.5	Proteasome subunit beta type 5_ACH85299.1	Protein degradation
Jack	4.20E-07	0.023216	11.82	2.9	Actin gamma_NP_001017750.1	Cell motility
Jack	4.11E-05	0.027859	4.22	2.2	Precerebellin protein_AAF04305.2	Inflammatory response
Jack	5.76E-07	0.01682	8.73	2.0	L-lactate dehydrogenase B_XP_014063047.1	ATP production
Jack	1.02E-07	0.010957	6.93	2.0	ARF GTPase protein GIT2_XP_013993786.1	Membrane re-modelling
Jack	3.48E-07	0.026799	3.41	2.0	Sex hormone-binding globulin β_ACJ25982.1	Hormone transport
Jack	2.82E-07	0.006442	2.93	1.9	3′,5′-Cyclic phosphodieserase_XP_014008493.1	cAMP hydrolysis
Jack	2.50E-05	0.043128	4.87	1.8	Ubiquitin hydrolase 44_XP_014063753.1	Protein de-ubiquitination
Jack	9.01E-05	0.036913	3.17	1.8	Creating kinase-2_ACH70915.1	ATP homeostasis
Jack	7.27E-09	0.005507	4.79	1.7	Proteasome subunit alpha type 4_AAH45970.1	Protein degradation

anions participating in spermatozoa membrane remodeling [80]. Future studies will be required to determine the significance of its elevated levels in hooknose males as it relates to sperm competition with jack males.

The importance of proteolysis within seminal plasma and sperm maturation is well documented and is crucial for stage specific regulation of spermatogenesis, removal of unviable or damaged spermatozoa, spermatozoa motility, and spermatozoa capacitation [81,82]. On the other hand, protease inhibitors attenuate unwanted proteolysis and maintain spermatozoa quiescence [81]. In our study, one of the most notable protease families present was the cathepsin proteases [83]. Cathepsins have been characterized in seminal plasma in a variety of species where they are proposed to function in membrane re-modeling and immunomodulation [83–85]. Three family members were identified in all biological replicates, including cathepsins O, K, and M. Cathepsin inhibitors cystatin and kininogen-1 were also identified (Table S1), indicating that regulated proteolysis is likely a prominent feature in Chinook salmon seminal plasma. In addition to cathepsin proteases, proteins involved in the ubiquitin-proteasome system (UPS) were also abundant in the seminal plasma of both tactics (Table S1). The extracellular UPS has garnered considerable attention recently in reproductive biology, and has been implicated in targeting proteins for degradation for the purpose of membrane re-modeling, removal of defective spermatozoa, and regulation of spermatozoa maturation [86]. Furthermore, there is growing evidence that measuring ubiquitin levels in seminal plasma is a viable clinical biomarker assay for infertility in humans, where higher levels correlate inversely with spermatozoa numbers, motility, and proper morphology [87]. Although we detected ubiquitin levels to be similar in our study (Table S1), there were UPS associated enzymes whose levels were different between the tactics. Most notably, the E3 ligase KEG-like enzyme was over two-fold higher in hooknose males (Fig. 7A, and Table 1). As E3 ligase enzymes are responsible for recognizing targets and presenting them to the ubiquitin conjugating machinery, elevated levels suggest that hooknose males may display a greater propensity to perform UPS-mediated activities. Supporting this hypothesis was the observation that the ubiquitin carboxy-terminal hydrolase 44-like enzyme, which is predicted to function as a deubiquitinase, was 1.8 fold higher in jack males than hooknose males. As the UPS system can exert multiple effects on spermatozoa, it will be interesting to examine how these protein differences in UPS-related enzymes affect sperm competition in the alternative reproductive tactics of Chinook salmon.

In conclusion, utilizing a high throughput label-free quantitative proteomics approach that incorporated ion mobility separation and data-independent acquisition, we identified known and novel seminal plasma proteins from Chinook salmon alternative reproductive tactics. Moreover, for the first time, quantitative proteomic differences between alternative reproductive tactics (hooknose and jack males) in seminal plasma were elucidated. Many of the protein abundance differences offer insight into the divergent reproductive strategies displayed by the Chinook salmon male tactics. Future work will now be centered on examining the physiological significance of the differential protein components and their mechanisms of action in relation to competitive and non-competitive fertilization success.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2017.01.019.

Transparency document

The transparency document associated with this article can be found, in online version.

Acknowledgements

This study was funded by Discovery Grants from the Natural Sciences and Engineering Research Council (NSERC) (06000-2015) of Canada to P.O.V. and T.E.P. T.E.P. was also supported by an Early Research Award (Ontario Ministry of Research and Innovation (ER11-08-208)). R.G. was supported by a NSERC CREATE (397997-2011) doctoral training grant. All research followed the University of Windsor and Canadian Council of Animal Care guidelines.

References

[1] R. Billard, Spermatogenesis and spermatology of some teleost fish species, Reprod. Nutr. Dev. 26 (1986) 877–920.
[2] P. Stockley, M.J. Gage, G.A. Parker, A.P. Moller, Sperm competition in fishes: the evolution of testis size and ejaculate characteristics, Am. Nat. 149 (1997) 933–954.
[3] M. Taborsky, R.F. Oliveira, H.J. Brockmann, The evolution of alternative reproductive tactics: concepts and questions, in: R.F. Oliveira, M. Taborsky, J.H. Brockmann (Eds.), Alternative Reproductive Tactics: An Integrative Approach, Cambridge University Press, Cambridge 2008, pp. 1–21.
[4] B.D. Neff, F. Peng, M.R. Gross, Sperm investment and alternative mating tactics in bluegill sunfish (*Lepomis macrochirus*), Behav. Ecol. 14 (2003) 634–641.
[5] G.A. Parker, Sperm competition games - sneaks and extra-pair copulations, P. Roy. Soc. Biol. Sci. 242 (1990) 127–133.
[6] M.J.G. Gage, P. Stockley, G.A. Parker, Effects of alternative male mating strategies on characteristics of sperm production in the Atlantic salmon (*Salmo salar*): theoretical and empirical investigations, Philos. T. Roy. Soc. B. 350 (1995) 391–399.
[7] T.V. Vladic, T. Jarvi, Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism, Proc. Biol. Sci. 268 (2001) 2375–2381.
[8] B. Berejikian, D.M. Van Doornik, J.J. Atkins, Alternative male reproductive phenotypes affect offspring growth rates in Chinook Salmon, T. Am. Fish. Soc. 140 (2011) 1206–1212.
[9] D.D. Heath, R.H. Devlin, J.W. Heath, G.K. Iwama, Genetic, environmental and interaction effects on the incidence of jacking in *Oncorhynchus-tshawytscha* (Chinook Salmon), Heredity 72 (1994) 146–154.
[10] E.W. Flannery, I.A.E. Butts, M. Slowinska, A. Ciereszko, T.E. Pitcher, Reproductive investment patterns, sperm characteristics, and seminal plasma physiology in alternative reproductive tactics of Chinook salmon (*Oncorhynchus tshawytscha*), Biol. J. Linn. Soc. 108 (2013) 99–108.
[11] I.A. Butts, O.P. Love, M. Farwell, T.E. Pitcher, Primary and secondary sexual characters in alternative reproductive tactics of Chinook salmon: associations with androgens and the maturation-inducing steroid, Gen. Comp. Endocrinol. 175 (2012) 449–456.
[12] R.R. Snook, Sperm in competition: not playing by the numbers, Trends Ecol. Evol. 20 (2005) 46–53.
[13] S.A. Ramm, L. McDonald, J.L. Hurst, R.J. Beynon, P. Stockley, Comparative proteomics reveals evidence for evolutionary diversification of rodent seminal fluid and its functional significance in sperm competition, Mol. Biol. Evol. 26 (2009) 189–198.
[14] S.A. Ramm, D.A. Edward, A.J. Claydon, D.E. Hammond, P. Brownridge, J.L. Hurst, R.J. Beynon, P. Stockley, Sperm competition risk drives plasticity in seminal fluid composition, BMC Biol. 13 (2015) 1–18.
[15] L. Locatello, F. Poli, M.B. Rasotto, Tactic-specific differences in seminal fluid influence sperm performance, Proc. R. Soc. Lond. B Biol. Sci. 280 (2013) 1–6.
[16] J.C. Silva, M.V. Gorenstein, G.Z. Li, J.P. Vissers, S.J. Geromanos, Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition, Mol. Cell. Proteomics 5 (2006) 144–156.
[17] R.P. Dator, K.W. Gaston, P.A. Limbach, Multiple enzymatic digestions and ion mobility separation improve quantification of bacterial ribosomal proteins by data independent acquisition liquid chromatography–mass spectrometry, Anal. Chem. 86 (2014) 4264–4270.
[18] N.J. Bond, P.V. Shliaha, K.S. Lilley, L. Gatto, Improving qualitative and quantitative performance for MS(E)-based label-free proteomics, J. Proteome Res. 12 (2013) 2340–2353.
[19] H. Niksirat, A. Kouba, P. Kozák, Post-mating morphological changes in the spermatozoon and spermatophore wall of the crayfish *Astacus leptodactylus*: insight into a non-motile spermatozoon, Anim. Reprod. Sci. 149 (2014) 325–334.
[20] H. Niksirat, P. James, L. Andersson, A. Kouba, P. Kozák, Label-free protein quantification in freshly ejaculated versus post-mating spermatophores of the noble crayfish *Astacus astacus*, J. Proteome 123 (2015) 70–77.
[21] S. Ghisaura, B. Loi, G. Biosa, M. Baroli, D. Pagnozzi, T. Roggio, S. Uzzau, R. Anedda, M.F. Addis, Proteomic changes occurring along gonad maturation in the edible sea urchin *Paracentrotus lividus*, J. Proteome 144 (2016) 63–72.
[22] M.A. Dietrich, G.J. Dietrich, A. Mostek, A. Ciereszko, Motility of carp spermatozoa is associated with profound changes in the sperm proteome, J. Proteome 138 (2016) 124–135.
[23] S.C. Stephen, Salmonine Introductions to the Laurentian Great Lakes, Canadian Special Publication of Fisheries and Aquatic Sciences, No. 132, NRC Research Press, 2001 36–47.
[24] T.E. Pitcher, B.D. Neff, MHC class IIB alleles contribute to both additive and nonadditive genetic effects on survival in Chinook salmon, Mol. Ecol. 15 (2006) 2357–2365.
[25] T.E. Pitcher, B.D. Neff, Genetic quality and offspring performance in Chinook salmon: implications for supportive breeding, Conserv. Genet. 8 (2007) 607–616.
[26] F. Finamore, F. Priego-Capote, F. Gluck, A. Zufferey, P. Fontana, J.-C. Sanchez, Impact of high glucose concentration on aspirin-induced acetylation of human serum albumin: an in vitro study, EuPA Open Proteomics. 3 (2014) 100–113.
[27] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. R. Stat. Soc. Ser. B Methodol. 57 (1995) 289–300.

[28] R Core Team, R: A Language and Environment for Statistical Computing, https://www.r-project.org 2016 (accessed 16/07/07).
[29] H. Wickham, ggplot: Elegant Graphics for Data Analysis, Springer-Verlag, New York, 2009.
[30] P.V. Shliaha, N.J. Bond, L. Gatto, K.S. Lilley, Effects of traveling wave ion mobility separation on data independent acquisition in proteomics studies, J. Proteome Res. 12 (2013) 2323–2339.
[31] K.A. Naish, R.B. Phillips, M.S. Brieuc, L.R. Newton, A.E. Elz, L.K. Park, Comparative genome mapping between Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*O. mykiss*) based on homologous microsatellite loci, G3: Genes|Genomes|Genetics 3 (2013) 2281–2288.
[32] P.J. Lester, S.J. Hubbard, Comparative bioinformatic analysis of complete proteomes and protein parameters for cross-species identification in proteomics, Proteomics 2 (2002) 1392–1405.
[33] A.J. Liska, A. Shevchenko, Expanding the organismal scope of proteomics: cross-species protein identification by mass spectrometry and its implications, Proteomics 3 (2003) 19–28.
[34] B. Habermann, J. Oegema, S. Sunyaev, A. Shevchenko, The power and the limitations of cross-species protein identification by mass spectrometry-driven sequence similarity searches, Mol. Cell. Proteomics 3 (2004) 238–249.
[35] J.G. Burniston, J. Connolly, H. Kainulainen, S.L. Britton, L.G. Koch, Label-free profiling of skeletal muscle using high-definition mass spectrometry, Proteomics 14 (2014) 2339–2344.
[36] B. Pilch, M. Mann, Large-scale and high-confidence proteomic analysis of human seminal plasma, Genome Biol. 7 (2006) 1–10.
[37] J. Nynca, G.J. Arnold, T. Frohlich, K. Otte, F. Flenkenthaler, A. Ciereszko, Proteomic identification of rainbow trout seminal plasma proteins, Proteomics 14 (2014) 133–140.
[38] M.A. Dietrich, G.J. Arnold, J. Nynca, T. Frohlich, K. Otte, A. Ciereszko, Characterization of carp seminal plasma proteome in relation to blood plasma, J. Proteome 98 (2014) 218–232.
[39] C.L. Harris, M. Mizuno, B.P. Morgan, Complement and complement regulators in the male reproductive system, Mol. Immunol. 43 (1–2) (2006) 57–67.
[40] A. Poiani, Complexity of seminal fluid: a review, Behav. Ecol. Sociobiol. 60 (2006) 289–310.
[41] M.A. Dietrich, M. Adamek, B. Bilinska, A. Hejmej, D. Steinhagen, A. Ciereszko, Characterization, expression and antibacterial properties of apolipoproteins A from carp (*Cyprinus carpio L.*) seminal plasma, Fish Shellfish Immunol. 41 (2014) 389–401.
[42] F.H. Pierik, J.T. Vreeburg, T. Stijnen, F.H. De Jong, R.F. Weber, Serum inhibin B as a marker of spermatogenesis, J. Clin. Endocrinol. Metab. 83 (1998) 3110–3114.
[43] A.E. O'Connor, D.M. De Kretser, Inhibins in normal male physiology, Semin. Reprod. Med. 22 (2004) 177–185.
[44] S.I. Buschow, B.W. van Balkom, M. Aalberts, A.J. Heck, M. Wauben, W. Stoorvogel, MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis, Immunol. Cell Biol. 88 (2010) 851–856.
[45] N.L. Anderson, M. Polanski, R. Pieper, T. Gatlin, R.S. Tirumalai, T.P. Conrads, T.D. Veenstra, J.N. Adkins, J.G. Pounds, R. Fagan, A. Lobley, The human plasma proteome: a nonredundant list developed by combination of four separate sources, Mol. Cell. Proteomics 3 (2004) 311–326.
[46] H.J. Glander, J. Kratzsch, C. Weisbrich, G. Birkenmeier, Insulin-like growth factor-I and alpha(2)-macroglobulin in seminal plasma correlate with semen quality, Hum. Reprod. 11 (1996) 2454–2460.
[47] A.A. Rehman, H. Ahsan, F.H. Khan, Alpha-2-Macroglobulin: a physiological guardian, J. Cell. Physiol. 228 (2013) 1665–1675.
[48] J.M. Vaughan, W.W. Vale, Alpha 2-macroglobulin is a binding protein of inhibin and activin, Endocrinology 132 (1993) 2038–2050.
[49] G. Raposo, W. Stoorvogel, Extracellular vesicles: exosomes, microvesicles, and friends, J. Cell Biol. 200 (2013) 373–383.
[50] J.M. Street, P.E. Barran, C.L. Mackay, S. Weidt, C. Balmforth, T.S. Walsh, R.T. Chalmers, D.J. Webb, J.W. Dear, Identification and proteomic profiling of exosomes in human cerebrospinal fluid, J. Transl. Med. 10 (2012) 5.
[51] D. Duijvesz, K.E. Burnum-Johnson, M.A. Gritsenko, A.M. Hoogland, M.S. Vredenbregt-van den Berg, R. Willemsen, T. Luider, L. Pasa-Tolic, G. Jenster, Proteomic profiling of exosomes leads to the identification of novel biomarkers for prostate cancer, PLoS One 8 (2013), e82589. .
[52] P.A. Gonzales, T. Pisitkun, J.D. Hoffert, D. Tchapyjnikov, R.A. Star, R. Kleta, N.S. Wang, M.A. Knepper, Large-scale proteomics and phosphoproteomics of urinary exosomes, J. Am. Soc. Nephrol. 20 (2009) 363–379.
[53] A. Bobrie, M. Colombo, G. Raposo, C. Thery, Exosome secretion: molecular mechanisms and roles in immune responses, Traffic 12 (2011) 1659–1668.
[54] X. Zhao, Y. Wu, J. Duan, Y. Ma, Z. Shen, L. Wei, X. Cui, J. Zhang, Y. Xie, J. Liu, Quantitative proteomic analysis of exosome protein content changes induced by hepatitis B virus in Huh-7 cells using SILAC labeling and LC-MS/MS, J. Proteome Res. 13 (2014) 5391–5402.
[55] R. Sullivan, F. Saez, J. Girouard, G. Frenette, Role of exosomes in sperm maturation during the transit along the male reproductive tract, Blood Cells Mol. Dis. 35 (2005) 1–10.
[56] K.G. Ronquist, B. Ek, A. Stavreus-Evers, A. Larsson, G. Ronquist, Human prostasomes express glycolytic enzymes with capacity for ATP production, Am. J. Physiol. Endocrinol. Metab. 304 (2013) E576–E582.
[57] L.L. Piehl, M.L. Fischman, U. Hellman, H. Cisale, P.V. Miranda, Boar seminal plasma exosomes: effect on sperm function and protein identification by sequencing, Theriogenology 79 (2013) 1071–1082.
[58] M. Alenquer, M.J. Amorim, Exosome biogenesis, regulation, and function in viral infection, Viruses. 7 (2015) 5066–5083.
[59] I.A. Rooney, J.P. Atkinson, E.S. Krul, G. Schonfeld, K. Polakoski, J.E. Saffitz, B.P. Morgan, Physiologic relevance of the membrane attack complex inhibitory protein CD59 in human seminal plasma: CD59 is present on extracellular organelles (prostasomes), binds cell membranes, and inhibits complement-mediated lysis, J. Exp. Med. 177 (1993) 1409–1420.
[60] A.S. Block, S. Saraswati, C.F. Lichti, M. Mahadevan, A.B. Diekman, Co-purification of Mac-2 binding protein with galectin-3 and association with prostasomes in human semen, Prostate 71 (2011) 711–721.
[61] A. Clayton, C.L. Harris, J. Court, M.D. Mason, B.P. Morgan, Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59, Eur. J. Immunol. 33 (2003) 522–531.
[62] O. Serang, J. Paulo, H. Steen, J.A. Steen, A non-parametric cutout index for robust evaluation of identified proteins, Mol. Cell. Proteomics 12 (2013) 807–812.
[63] L. Huang, S.I. Wickramasekara, T. Akinyeke, B.S. Stewart, Y. Jiang, J. Raber, C.S. Maier, Ion mobility-enhanced MSE-based label-free analysis reveals effects of low-dose radiation post contextual fear conditioning training on the mouse hippocampal proteome, J. Proteome 140 (2016) 24–36.
[64] C. Selby, Sex hormone binding globulin: origin, function and clinical significance, Ann. Clin. Biochem. 27 (1990) 532–541.
[65] L. Lazaros, N. Xita, A. Kaponis, K. Zikopoulos, N. Sofikitis, I. Georgiou, Evidence for association of sex hormone-binding globulin and androgen receptor genes with semen quality, Andrologia 40 (2008) 186–191.
[66] C.A. Heinlein, C. Chang, The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions, Mol. Endocrinol. 16 (2002) 2181–2187.
[67] S. Tardif, O.A. Madamidola, S.G. Brown, L. Frame, L. Lefievre, P.G. Wyatt, C.L. Barratt, S.J. Martins Da Silva, Clinically relevant enhancement of human sperm motility using compounds with reported phosphodiesterase inhibitor activity, Hum. Reprod. 29 (2014) 2123–2135.
[68] L. Lefievre, E. De Lamirande, C. Gagnon, The cyclic GMP-specific phosphodiesterase inhibitor, sildenafil, stimulates human sperm motility and capacitation but not acrosome reaction, J. Androl. 21 (2000) 929–937.
[69] D.D. Hoskins, M.L. Hall, D. Munsterman, Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors and seminal plasma, Biol. Reprod. 13 (1975) 168–176.
[70] M. Morisawa, K. Ishida, Short-term changes in levels of cyclic AMP, adenylate cyclase, and phosphodiesterase during the initiation of sperm motility in rainbow trout, J. Exp. Zool. 242 (1987) 199–204.
[71] P. Dzeja, A. Terzic, Adenylate kinase and AMP signaling networks: metabolic monitoring, signal communication and body energy sensing, Int. J. Mol. Sci. 10 (2009) 1729–1772.
[72] F. Lahnsteiner, B. Berger, T. Weismann, R.A. Patzner, Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism, Fish Physiol. Biochem. 15 (1996) 167–179.
[73] F. Lahnsteiner, B. Berger, T. Weismann, R.A. Patzner, Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism, Aquaculture 163 (1998) 163–181.
[74] W. Cao, L. Haig-Ladewig, G.L. Gerton, S.B. Moss, Adenylate kinases 1 and 2 are part of the accessory structures in the mouse sperm flagellum, Biol. Reprod. 75 (2006) 492–500.
[75] C. Burgos, C. Maldonado, N.M. Gerez de Burgos, A. Aoki, A. Blanco, Intracellular localization of the testicular and sperm-specific lactate dehydrogenase isozyme C4 in mice, Biol. Reprod. 53 (1995) 84–92.
[76] F. Odet, S.A. Gabel, J. Williams, R.E. London, E. Goldberg, E.M. Eddy, Lactate dehydrogenase C and energy metabolism in mouse sperm, Biol. Reprod. 85 (2011) 556–564.
[77] J.L. Fitzpatrick, P.M. Craig, C. Bucking, S. Balshine, C.M. Wood, G.B. McClelland, Sperm performance under hypoxic conditions in the intertidal fish *Porichthys notatus*, Can. J. Zool. 87 (2009) 464–469.
[78] W.C. Ford, Regulation of sperm function by reactive oxygen species, Hum. Reprod. Update 10 (2004) 387–399.
[79] L. Yan, J. Liu, S. Wu, S. Zhang, G. Ji, A. Gu, Seminal superoxide dismutase activity and its relationship with semen quality and SOD gene polymorphism, J. Assist. Reprod. Genet. 31 (2014) 549–554.
[80] T. Kobayashi, T. Miyazaki, M. Natori, S. Nozawa, Protective role of superoxide dismutase in human sperm motifity: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa, Hum. Reprod. 6 (1991) 987–991.
[81] B.A. Laflamme, M.F. Wolfner, Identification and function of proteolysis regulators in seminal fluid, Mol. Reprod. Dev. 80 (2013) 80–101.
[82] S.S. Chung, L.J. Zhu, M.Y. Mo, B. Silvestrini, W.M. Lee, C.Y. Cheng, Evidence for cross-talk between Sertoli and germ cells using selected cathepsins as markers, J. Androl. 19 (1998) 686–703.
[83] V. Turk, V. Stoka, O. Vasiljeva, M. Renko, T. Sun, B. Turk, D. Turk, Cysteine cathepsins: from structure, function and regulation to new frontiers, Biochim. Biophys. Acta 1824 (2012) 68–88.
[84] S. Conus, H.U. Simon, Cathepsins and their involvement in immune responses, Swiss Med. Wkly. 140 (2010) w13042.
[85] C. Bonnans, J. Chou, Z. Werb, Remodelling the extracellular matrix in development and disease, Nat. Rev. Mol. Cell Biol. 15 (2014) 786–801.
[86] R. Bose, G. Manku, M. Culty, S.S. Wing, Ubiquitin-proteasome system in spermatogenesis, Adv. Exp. Med. Biol. 759 (2014) 181–213.
[87] P. Sutovsky, R. Hauser, M. Sutovsky, Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology laboratory clinic population, Hum. Reprod. 19 (2004) 628–638.