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Proteomic analysis of pikeperch seminal plasma provides novel insight into the testicular development of domesticated fish stocks

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ABSTRACT

Control of the reproduction of domesticated stocks is considered a prerequisite for aquaculture development of pikeperch. However, knowledge about the physiology of the captive pikeperch male reproductive system and the biology of semen is very limited, especially regarding protein characteristics. The aims of our study were to characterize pikeperch sperm quantity and quality parameters and to analyze changes in the proteome of the same males spawned for the first and second times. Moreover, attempts were made to generate the first proteomic library of seminal plasma proteins. Semen collected during the first spawning season was characterized by lower sperm concentration and volume than for the second season. Using mass spectrometry-based label-free quantitative proteomics, we identified 850 proteins in the seminal plasma of pikeperch from both spawning seasons, and 65 seminal proteins were found to be differentially abundant between the first and second spawning seasons. The majority of differentially abundant proteins were involved in stress and immune responses, developmental processes, cofactor metabolic processes, proteolysis, cellular oxidant detoxification and organization of the extracellular matrix (**ECM**). In addition, several proteins unique to pikeperch seminal plasma were identified, including antifreeze proteins, hibernation-specific plasma proteins, lectins and vitellogenin. In summary, our results indicate that males that spawned for the first time were characterized by incompletely mature gonads and the expression of proteins associated with the early phase of spermatogenesis and ECM organization. On the other hand, males that spawned for the second time exhibited advanced gonadal maturation and expression of proteins related to the late stage of spermatogenesis and sperm maturation, including regulation of reactive oxygen species generation, bicarbonate production, sperm elongation and separation. The identification of a large number of seminal plasma proteins provides a valuable resource for understanding the functions of seminal plasma and the molecular mechanisms involved in testicular development and maturation in domesticated fish, which is a prerequisite for better control of reproduction in captivity.

Implications

In this study, we compared sperm quality parameters and proteomic profile of the seminal plasma obtained from cultured pikeperch over their first and second spawning. We found that first-time spawners were characterized by lower performance what coincided with differential abundance of many important seminal plasma proteins. It indicates, that first-time spawners should be carefully reconsidered before being used for selective breeding programs due to their immaturity. Besides, proteomic profiling of seminal plasma provides significant insight into the mechanisms involved in testicular development and maturation, shedding light on the proteins involved in stress and the immune response, developmental processes, detoxification and spermatogenesis.

Introduction

Pikeperch or zander (*Sander lucioperca* L.) belongs to the percid family and has become one of the most important species for the

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diversification of European aquaculture. This species has excellent meat quality, good growth performance and market acceptance (Policar et al., 2019). The main role of pikeperch in the ecosystem of open waters is to regulate the populations of small cyprinids, leading to improved water quality. At present, the increasing market demand for pikeperch is supplied by wild catches, which has become an important component of the serious decline of many populations. This situation has created a need for restocking programs, which are also based on the reproduction of wild-caught breeders. Successful full-life-cycle rearing of this species in recirculation aquaculture systems (**RASs**) is an alternative to preserve wild pikeperch populations and to promote feasible and sustainable aquaculture (Policar et al., 2019).

Fish under domestication processes are exposed to highly modified living conditions controlled by humans, such as water quality, food quality, and photothermal conditions (Teletchea and Fontaine, 2014). In addition, intensive culture conditions (such as RAS) are perceived as important stressors since fish are subjected to unnatural confinement and frequent handling (Sarameh et al., 2012). Furthermore, breeders are often exposed to specific reproductive procedures, such as hormonal stimulation, which has modulatory effects on the physiology of fish and their reproductive effectiveness (Żarski et al., 2019 and 2020). Domestication has been found to negatively affect the hatching rate as well as embryo survival and increase the malformation of larvae (Khendek et al., 2018). Therefore, control over the reproduction of domesticated stocks is considered a prerequisite for aquaculture development in pikeperch. Although this species has been extensively studied to improve production efficiency under culture conditions, knowledge about the physiology of the male reproductive system and the biology of semen is still limited and mainly concerns the characterization of sperm quality parameters (sperm motility and concentration) in response to hormonal stimulation, different rearing conditions, sperm-activating media and the analysis of sperm ultrastructure and morphology (Blecha et al., 2015; Sarosiek et al., 2016). However, there is limited information concerning the composition of pikeperch seminal plasma (Nynca et al., 2010), which might be a rich source of biomarkers of reproductive physiology and/or disorders in male fishes.

The overall role of fish seminal plasma is to create an optimal environment for the maturation and storage of spermatozoa in the reproductive system. Furthermore, seminal plasma can be potentially important during external fertilization to create a microenvironment for gamete meetings (Billard, 1983). The application of comprehensive proteomic approaches has allowed us to gain new insights into the functions of fish seminal plasma proteins in the protection of spermatozoa and reproductive tract tissues against infection and oxidative stress, energy resources and maintenance of the specific membrane composition of spermatozoa (Nynca et al., 2014; Dietrich et al., 2014). It must be emphasized that the composition of seminal plasma proteins varies among species, likely reflecting species-specific characteristics of reproduction in fish. Since seminal plasma proteins exert an important effect on sperm function and structure, in-depth characterization of the protein profile of pikeperch seminal plasma is a prerequisite to better understand the specific functions of seminal plasma proteins in the pikeperch reproductive tract. This knowledge is a prerequisite for the development and improvement of methods of controlled reproduction in pikeperch.

In several species, the first sexual maturation or onset of spermatogenesis of captive fish stagnates for overextended periods. In pikeperch, successful induction and control of gonadal development must involve an extended (3–5 months) wintering period with temperatures below 10 °C (Żarski et al., 2019). Therefore, in hatchery practice, a photothermal program simulating annual fluctuations is applied (Fontaine et al., 2015). Apart from the photothermal regime, other factors, such as age, size and the rank of spawning, may be responsible for successful gonadal development. Pikeperch that reproduce for the first time may exhibit lowered reproductive performance (Khendek et al., 2018). Obtaining good-quality spermatozoa is of crucial importance for the feasibility of controlled reproduction and for successful selective breeding programs (Policar et al., 2019). However, obtaining good-quality semen from domesticated pikeperch stocks remains challenging, which is well reflected in the high intragroup variability of the quality of sperm obtained (Żarski et al., 2020). This can be linked with improper environmental conditions, breeding procedures and – presumably – selection of the spawners, which is usually done following the first spawning operation. However, considering that the first spawning attempt of RAS-grown pikeperch breeders may suffer from considerable impairments (Khendek et al., 2018), it is of high importance to acquire knowledge of reproductive system characteristics in subsequent spawning seasons, starting from the first spawning attempt. This will allow a better understanding of the reproduction of pikeperch in captivity.

We addressed this knowledge gap using seminal plasma proteomics in pikeperch, hypothesizing that the second spawning season causes profound changes in the semen quality and proteome of seminal plasma, especially in proteins associated with spermatogenesis and sperm maturation. The aims of our study were to characterize sperm quantity and quality parameters and to analyze changes in the proteome of the same pikeperch males spawned for the first and second time using a label-free proteomic strategy. Moreover, attempts at generating the first proteomic library were undertaken to better understand the physiological processes underlying gonadal development and the function of seminal plasma in this species.

Material and methods

Source of gametes

The experiment was designed to reflect commercial practices in intensive aquaculture of pikeperch, which involves first induction of gonadal cycle and spawning of fish grown at constant photothermal conditions (long photo-phase [>14 h] and high temperature [>20 °C]) ensuring high growth of fish accompanied with photothermally induced retardation of gonadal development (for details see Fontaine et al., 2015). In commercial practice such fish are subsequently, year-to-year, used for reproduction following exposure to annual photothermal fluctuations indispensable for proper gonadogenesis (Fontaine et al., 2015). In this way, our aim was to compare the proteomic seminal plasma profile in first-time spawners (having their gonadal development completed for the first time) with their second spawning act. Fish (age 2+, weight 980 ± 115 g) were obtained in 2017 from the AquaPri (Denmark) farm and handled as described by Judycka et al. (2021). After acclimation period (4 weeks, 15 °C), seven fish were randomly sacrificed before the experiment in order to confirm their immaturity (five out of seven fish were found to be males; all males had underdeveloped gonads with gonadosomatic index lower than 0.1% of BW, being typically observed in pikeperch males prior to the induction of gonadal cycle; Fontaine et al., 2015; Khendek et al., 2018). The fish were then exposed to a 6-month-long photothermal program (as shown in Fig. 1) aimed at induction of the gonadal cycle and spawning. This led to the first spawning event of the broodstock in late May 2018, during which the first sperm collection occurred. Fish were tagged with PIT tags (passive integrated transponders) in the cheek muscle. Then, the fish were again exposed to the specific photothermal program (Fig. 1), which

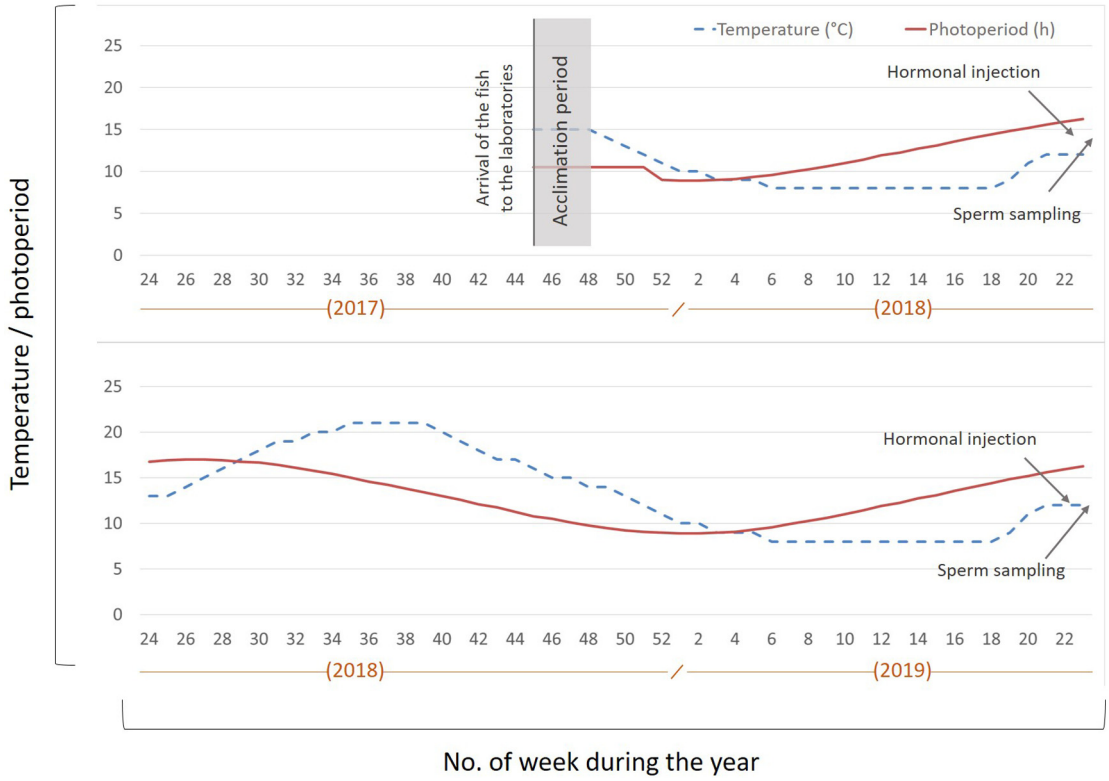


Fig. 1. Photothermal program applied to pikeperch broodstock during the realization of the study.

led to the second sperm collection performed in late May – early June 2019. Feeding of fish and photoperiod were as described by Żarski et al. (2019). The males were stimulated with gonadotropin releasing hormone analog (sGnRHa) using procedure described by Żarski et al. (2020) and Judycka et al. (2021). Proteomic analysis was performed on the seminal plasma of seven individual males during the first spawning (age 3+, weight 1 494 ± 350 g) and the same seven males during the second spawning season (age 4+, weight 1 688 ± 402 g).

Measurement of semen quality parameters

The percentage of motile spermatozoa was examined with computer-assisted sperm analysis using the CRISMAS system (Image House Ltd., Copenhagen, Denmark). Semen was activated using the procedure described by Judycka et al. (2021). The total volume of semen (ml) was measured directly after collection using a pipette (0.01 ml accuracy). The sperm concentration of fresh semen was measured using a NucleoCounter SP-100 computer-aided fluorescence microscope (Chemometec, Allerød, Denmark) using the procedure described by Judycka et al. (2021). Total sperm production (sperm concentration × semen volume) and male BW were used to estimate the total number of sperm per kg of BW of fish (10^9 spermatozoa/kg BW).

Measurement of seminal plasma osmolality and protein concentration

Seminal plasma was obtained by centrifugation of semen at 5 000*g* (4 °C, 15 min), and then the supernatant was centrifuged again at 8 000*g* (4 °C, 10 min). Seminal plasma osmolality was measured using a Minitübe Abfüll-u Labortechnik Löser apparatus (Tiefenbach, Germany). The protein concentration was measured by the method of Bradford using a Coomassie Plus Kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as a standard.

Label-free protein quantification, identification of seminal plasma proteins and statistical analysis

Sample preparation for mass spectrometry analysis

Each protein sample (50 μg) was diluted to 200 μl with 50 mM Tris-HCl, pH 8.0. Disulfide bonds were reduced by a 1 h incubation with 20 mM tris(2-carboxyethyl)phosphine at 60 °C followed by cysteine blockage using 50 mM methyl methanethiosulfonate. After overnight protein digestion at 37 °C with 2 μg of trypsin/LysC mix (Promega, WI, USA), peptides were diluted to 1 ml with 0.1% formic acid (**FA**) and cleaned using 10 mg Oasis hydrophilic-lipophilic balanced columns (Waters, Milford, MA, USA). Briefly, cartridges were conditioned with methanol and MS-grade water. After sample loading and rinsing with 1 ml of water, peptides were eluted from the column with 70% acetonitrile (**ACN**) and 0.1% FA. Aliquots were dried and resuspended in 60 μl of extraction solution (2% ACN, 0.1% trifluoroacetic acid). To ensure equal sample loading on the HPLC column, peptide concentrations were measured using a Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific).

Mass spectrometry

Three micrograms of peptides from each sample was analyzed using a liquid chromatography–mass spectrometry (**LC–MS**) system composed of a UPLC chromatograph (nanoAcquity, Waters) coupled to a Q Exactive mass spectrometer (Thermo Scientific). Peptides were trapped on a C18 precolumn (180 μm × 20 mm, Waters) using 0.1% FA in water as a mobile phase and further separated on a nanoAcquity ethylene bridged hybrid C18 column (75 μm × 250 mm, 1.7 μm, Waters) using an ACN gradient (0–35% ACN in 160 min) in the presence of 0.1% FA at a flow rate of

250 nl/min. Data acquisition was carried out using a data-dependent method with the top 12 precursors selected for second mass spectra analysis after collisional induced fragmentation with a normalized collision energy of 27%. Full MS scans covering the mass range of 300–2 000 were acquired at a resolution of 70 000 with a maximum injection time of 60 ms and an automated gain control (**AGC**) target value of 1-e6. Second mass spectra scans were acquired with a maximum injection time of 60 ms and an AGC target value of 5-e5 with an isolation window of 3.0 m/z. Dynamic exclusion was set to 30 s.

Analysis of mass spectrometry data

The acquired tandem mass spectrometry (MS/MS) data were preprocessed with Mascot Distiller software (v. 2.7, MatrixScience, London, UK), and a search was performed with the Mascot Search Engine (MatrixScience, Mascot Server 2.7) against the *S. lucioperca* proteins (56 731 sequences; 44 537 686 residues) deposited in the National Center for Biotechnology Information database (version from 7 November 2020). The peptide and fragment mass tolerance settings were established separately for individual LC–MS/MS runs after a measured mass recalibration, as described previously (Malinowska et al., 2012), based on the results of a 1st pass peptide/protein search, resulting in a parent ion mass tolerance of 5 ppm and fragment mass tolerance of 0.01 Da. The rest of the search parameters were as follows: enzyme, trypsin; missed cleavages, 1; fixed modifications, methylthio (C); variable modifications, oxidation (M); instrument, higher-energy C-trap dissociation. A statistical assessment of the confidence of peptide assignments was based on the target/decoy database search strategy (Elias et al., 2005). This procedure provided q-value estimates for each peptide spectrum match in the data set. All queries with *q*-values > 0.01 were removed from further analysis, as well as proteins with less than two peptides and proteins identified by a subset of peptides from another protein. The mass calibration and data filtering described above were carried out with MScan software, developed in-house (http://proteom.ibb.waw.pl/mscan/).

Quantification and statistical analysis of proteomic data

The lists of identified peptides were merged into one common list. This list was overlaid onto 2-D heatmaps generated from LC–MS profile datasets by tagging the peptide-related isotopic envelopes with corresponding peptide sequence tags on the basis of the measured/theoretical mass difference, the deviation from the predicted elution time and the match between theoretical and observed isotopic envelopes. The abundance of each peptide was determined as the volume of a 2-D fit to the monoisotopic peak of the tagged isotopic envelope. Quantitative values were next exported into text files, along with peptide/protein identifications, for statistical analysis with Diffprot (Malinowska et al., 2012) software. To select differentially abundant proteins (**DAPs**) between seminal plasma of semen collected during the first and second spawning seasons, a paired version of Diffprot test statistics was run with the following parameters: number of random peptide sets = 10^6; clustering of peptide sets – only when 90% identical; normalization by LOWESS.

Bioinformatic analysis

All the proteins that met the criteria for being differentially expressed were compared by hierarchical cluster analysis using the Cluster 3.0 program. Hierarchical clustering, volcano plots and principal component analysis (**PCA**) were performed with the R suite. To identify significantly enriched Gene Ontology (**GO**) functional groups of DAPs, GO annotation was performed using g:Profiler software. GO analysis for all identified seminal plasma proteins was carried out using three groups of protein annotations: cellular component, biological process, molecular function, Kyoto Encyclopedia of Genes and Genomes (**KEGG**) and Reactome pathways.

The protein–protein interactions were predicted using the Search Tool for the Retrieval of Interacting Genes (STRING, version 9.1) (http://string-db.org), which determines both physical and functional associations between proteins. The subset of 64 proteins that showed significant differential abundance between both seasons was entered into the STRING database. The *Homo sapiens* orthologs were determined within the STRING database from each *S. lucioperca* amino acid sequence. Default settings were used, with the interaction score set to medium confidence (0.4). Each node represents a protein, while the edges indicate the strength of the relationship between proteins (i.e., more edges give higher confidence).

Statistical analysis

Data analysis of semen characteristics was performed using GraphPad Prism software v. 8.4.3 (GraphPad Software Inc. San Diego, CA, USA). For statistical procedures, the percentage data of sperm motility were normalized by arcsine square root transformation. The results are presented as the mean ± SD. Paired Student's t-tests were performed to assess the significance of semen characteristics between the 1st and 2nd spawning events. Comparisons yielding a value of $P < 0.05$ were regarded as statistically significant.

Results

Semen characteristics

Semen collected during the second spawning was characterized by approximately two times higher sperm volume, four times higher sperm concentration and six times higher total number of sperm/kg BW than for the first spawning (Fig. 2A–C). On the other hand, the percentage of sperm motility did not differ between spawning seasons (Fig. 2D). The protein concentration of seminal plasma of semen collected during the second spawning was two times higher (Fig. 2E), whereas osmolality was 6% lower than that of semen collected during the first spawning (Fig. 2F).

Differential protein profile of seminal plasma of semen collected during the first and second spawning

A total of 14 297 peptides belonging to 1 291 proteins (Supplementary Material S1) were identified from the combined 14 LC–MS/MS runs. The total number of protein counts for each sample varied from 1 023 to 806. Out of 1 291, 850 (~66%) proteins were present in at least 50% of seminal plasma samples collected during the first and second spawning. The average and median intergroup interquartile ranges were markedly higher than intragroup values (average intergroup interquartile was 0.52 and 55 for 1st and 2nd spawning season, respectively while average intragroup interquartile was 0.66). PCA identified five dimensions, of which the first two explained 57.9% of the cumulative variance. PCA score plots showed good separation of seminal plasma proteins of semen collected during the 1st and 2nd spawning season (Fig. 3A). Among the identified proteins, 65 were found to be differentially abundant. Compared to the 1st spawning, 31 proteins were upregulated and 34 proteins were downregulated in seminal plasma collected during the 2nd spawning (Fig. 3B, C, Table 1). Nine upregulated and 15 downregulated proteins in the 2nd spawning were characterized by a fold change greater than two (Table 1).

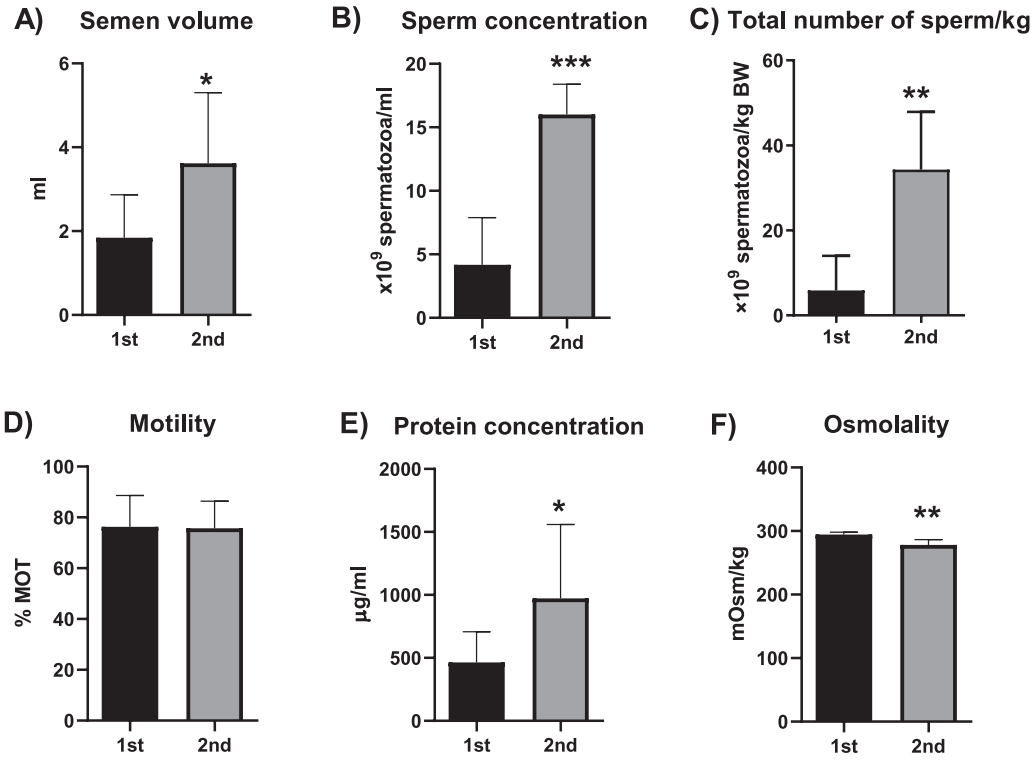


Fig. 2. Characteristics of pikeperch semen collected during the first and second spawning seasons (n = 7 in each group): semen volume (A), sperm concentration (B), total number of sperm/kg BW of fish, C), sperm motility (D) and seminal plasma protein concentration (E) and seminal plasma osmolality (F). The results are expressed as the means ± SD. Differences between parameters of semen collected during 1st and 2nd spawning seasons are indicated as *, ** and *** for $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

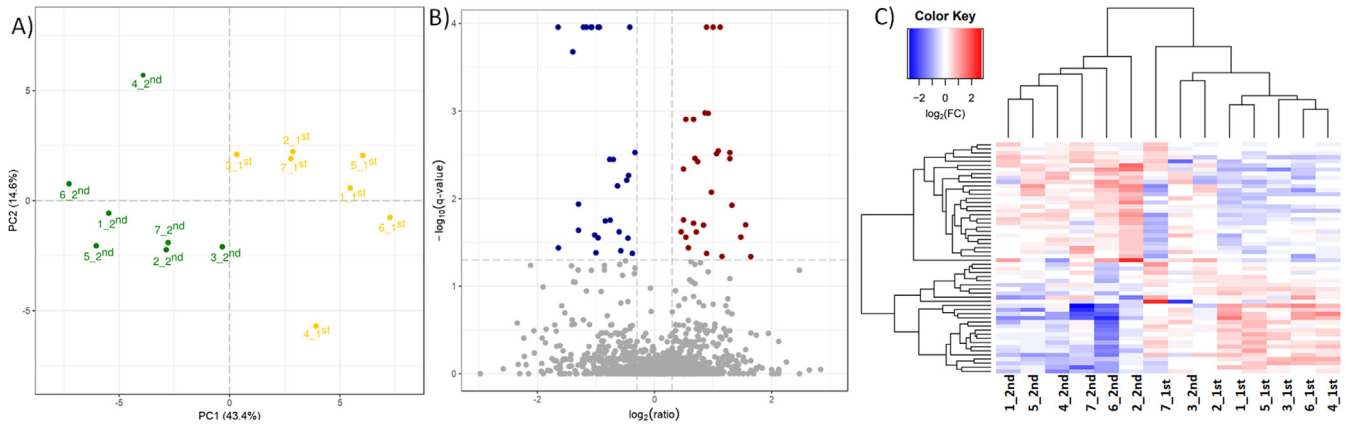


Fig. 3. Principal component (PC) analysis (A) and differential abundance analysis (B, C) for pikeperch seminal plasma proteins collected during the first and second spawning.

Functional analysis of differentially abundant proteins

Functional enrichment analysis of DAPs identified stress and immune responses including leukocyte-mediated immunity and inflammatory response, developmental process, cofactor metabolic process, proteolysis, cellular oxidant detoxification and extracellular matrix organization, as the most represented GO biological processes (Table 2). Of the GO molecular function terms, antioxidant activity, peptidase inhibitor activity, enzyme regulator activity, peptidase activity, ubiquitin protein ligase binding, L-malate dehydrogenase activity, calcium ion binding and protein folding chaperone were enriched for DAPs. The majority of DAPs were annotated as localized to the extracellular region, including exosomes and extracellular matrix. The remaining proteins were localized to the endomembrane system and cell membrane. KEGG analysis indicated two immunological pathways (complement and coagulation cascade, antigen processing and presentation) and cysteine and methionine metabolism. Reactome analysis indicated that chaperone-mediated autophagy, post-translational protein phosphorylation and the innate immune system were enriched pathways among DAPs.

Online STRING software was applied to construct the protein-protein interaction network of seminal plasma proteins altered during the first and second reproductive seasons (Fig. 4). The

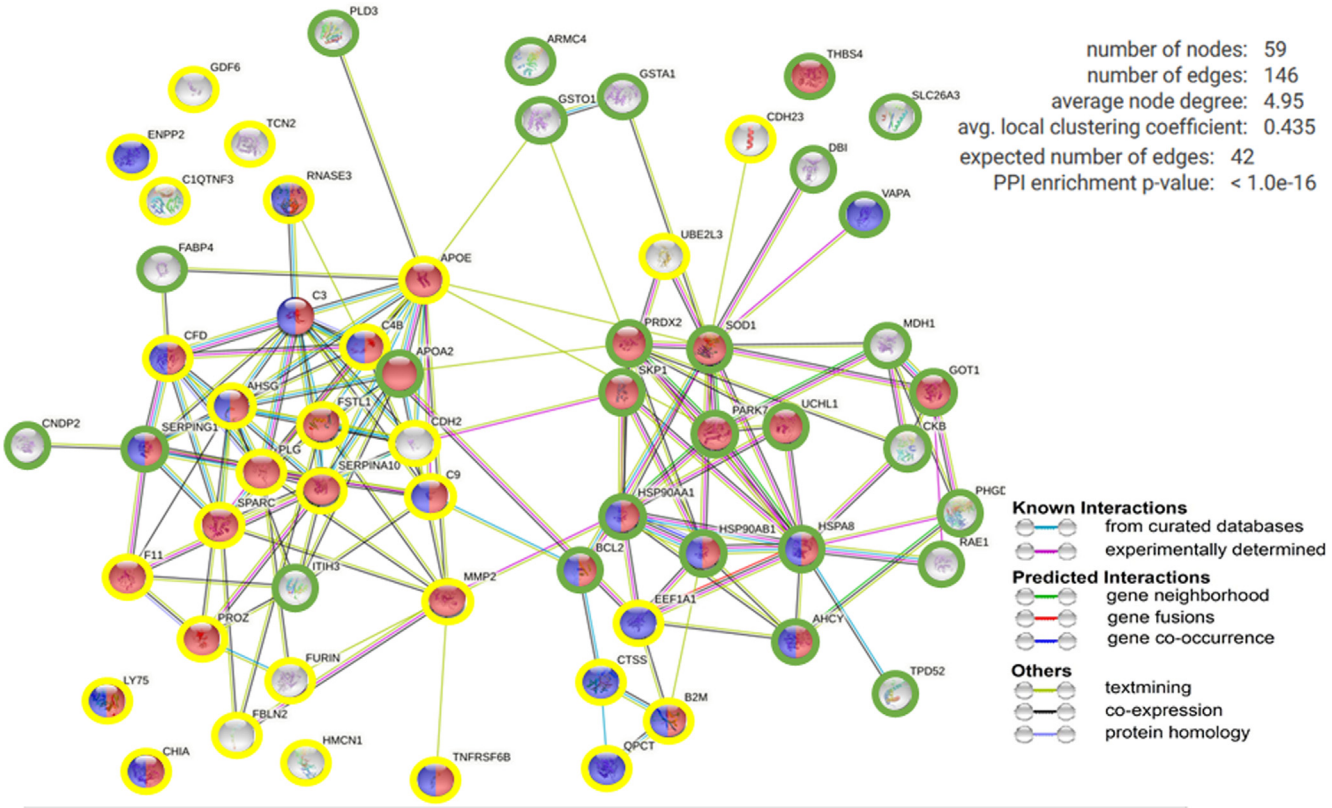
Table 1
Differentially abundant proteins in seminal plasma of pikeperch semen collected during the first and second spawning seasons.

No	Protein ID	Accession number	Gene ID	*P*-value	FC 2nd/1st
1	Bcl-2-like protein 13 isoform X2	XP031141331.1	*BCL2*	0.00408	3.48
2	D-3-phosphoglycerate dehydrogenase	XP031153860.1	*PGDH3*	0.01193	3.26
3	Aspartate aminotransferase, cytoplasmic	XP031161007.1	*GOT1*	0.03198	2.93
4	Ubiquitin carboxyl-terminal hydrolase isozyme L1	XP031132893.1	*UCHL1*	0.00865	2.64
5	VAMP (vesicle-associated membrane protein)-associated protein A, like	XP031149619.1	*VAPA*	0.01165	2.61
6	Uncharacterized protein pld7 isoform X1	XP031135889.1	*PLD3*	0.03108	2.40
7	S-phase kinase-associated protein 1	XP031163837.1	*SKP1*	0.00260	2.37
8	Creatine kinase, brain a isoform X3	XP031136267.1	*CKB*	0.00012	2.21
9	Armadillo repeat-containing protein 4 isoform X2	XP035854147.1	*ARMC4*	0.00230	2.05
10	Fatty acid binding protein 4a	XP031152114.1	*FABP4*	0.00781	1.99
11	Superoxide dismutase [Cu-Zn]	XP031136720.1	*SOD1*	0.00195	1.94
12	mRNA export factor isoform X2	XP031134510.1	*RAE1*	0.04533	1.92
13	Heat shock protein HSP 90-beta isoform X1	XP031178366.1	*HSP90AB1*	0.00020	1.89
14	Heat shock protein family A (Hsp70) member 8b	XP031152270.1	*HSPA8B*	0.00179	1.85
15	Parkinson disease protein 7 homolog	XP031157936.1	*PARK7*	0.04539	1.77
16	Glutathione S-transferase, alpha tandem duplicate 1	XP031159573.1	*GSTA1*	0.01344	1.74
17	Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	XP031177618.1	*HSP90AA1*	0.03973	1.72
18	Adenosylhomocysteinase	XP031157229.1	*AHCY*	0.00045	1.66
19	Acyl-CoA-binding protein	XP031145495.1	*DBI*	0.04688	1.66
20	Cytosolic non-specific dipeptidase-like	XP031151998.1	*CNDP2*	0.00907	1.60
21	Heat shock cognate 71 kDa protein	XP031166541.1	*HSPA8*	0.01450	1.59
22	Plasma protease C1 inhibitor	XP031158599.2	*SERPING1*	0.00012	1.58
23	Tumor protein D52 isoform X3	XP031152123.1	*TPD52*	0.00211	1.58
24	Peroxiredoxin-2	XP031140279.1	*PRDX2*	0.00331	1.57
25	Inter-alpha-trypsin inhibitor heavy chain H3	XP031134097.1	*ITIH3*	0.00229	1.56
26	Glutathione S-transferase omega-1	XP031162938.1	*GSTO1*	0.04697	1.50
27	Malate dehydrogenase, cytoplasmic isoform X1	XP031158555.1	*MDH1*	0.00907	1.48
28	Uncharacterized protein LOC116064680 similar to saxitoxin and tetrodotoxin-binding protein 1-like isoform X2	XP035848828.1	*fish specific*	0.00774	1.44
29	Chloride anion exchanger	XP031162075.1	*SLC26A3*	0.00812	1.33
30	Complement C3-like isoform X1	XP031145027.1	*C3*	0.00908	1.36
31	Apolipoprotein A-II	XP035862199.1	*APOA2*	0.00328	1.08
32	SPARC	XP031172982.1	*SPARC*	0.00681	−3.57
33	Elongation factor 1-alpha	XP031151518.1	*EEF1A1*	0.04910	−3.33
34	Thrombospondin-4-B	XP031173651.2	*THBS4*	0.00012	−3.21
35	Ubiquitin-conjugating enzyme E2 L3a	XP031157067.1	*UBE2L3*	0.01607	−3.04
36	Coagulation factor XI-like	XP031179016.1	*F11*	0.00012	−2.96
37	Ribonuclease-like 3	XP035862503.1	*RNASE3*	0.00747	−2.81
38	Beta-2-microglobulin-like	XP031144067.1	*B2M*	0.00012	−2.70
39	Glutaminyl-peptide cyclotransferase	XP031144511.1	*QPCT*	0.00666	−2.66
40	Protein Z-dependent protease inhibitor-like	XP031170926.1	*SERPINA10*	0.00748	−2.62
41	Cadherin-2	XP031150057.1	*CDH2*	0.00020	−2.49
42	Gonadal somatic cell derived factor	XP031173515.1	*GDF6*	0.00012	−2.35
43	Acidic mammalian chitinase-like	XP031146118.2	*CHIA*	0.00748	−2.21
44	Alpha-2-HS-glycoprotein-like	XP031167679.2	*AHSG*	0.00857	−2.12
45	Complement C1q tumor necrosis factor-related protein 3-like	XP031135812.1	*C1QTNF3*	0.00012	−2.10
46	Protein Z, vitamin K-dependent plasma glycoprotein a	XP031168627.1	*PROZ*	0.00739	−2.05
47	Furin a	XP035855332.1	*FURIN*	0.00012	−1.93
48	Galactose-specific lectin nattectin-like	XP031170500.1	*fish specific*	0.01084	−1.90
49	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 isoform X4	XP035862693.1	*ENPP2*	0.00012	−1.89
50	Tumor necrosis factor receptor superfamily member 6B-like	XP035848954.1	*TNFRSF6B*	0.00012	−1.81
51	Cathepsin S-like	XP031154340.1	*CTSS*	0.02251	−1.73
52	Fibulin-2	XP035857650.1	*FBLN2*	0.00050	−1.72
53	Follistatin-related protein 1	XP031151374.1	*FSTL1*	0.00027	−1.70
54	Apolipoprotein Eb	XP031144808.1	*APOE*	0.04931	−1.67
55	Hemicentin-1-like	XP035849716.1	*HMCN1*	0.00818	−1.65
56	Uncharacterized protein LOC116056820 similar to saxitoxin and tetrodotoxin-binding protein 1 90% identity	XP035849959.1	*fish specific*	0.00211	−1.59
57	72 kDa type IV collagenase	XP031137366.1	*MMP2*	0.00227	−1.52
58	Complement component C9 isoform X1	XP031173480.1	*C9*	0.00175	−1.49
59	Complement C4B	XP031154830.1	*C4B*	0.00231	−1.45
60	Cadherin-23-like	XP035857333.1	*CDH23*	0.01408	−1.43
61	Lymphocyte antigen 75	XP031162178.1	*LY75*	0.00040	−1.38
62	Transcobalamin-2	XP031164739.1	*TCN2*	0.02865	−1.37
63	Plasminogen	XP031169706.1	*PLG*	0.00012	−1.34
64	Complement factor D	XP035850587.1	*CFD*	0.00507	−1.33
65	Complement C3-like isoform X1	XP031145028.1	*C3*	0.00040	−1.30

FC = fold change; a positive ratio denotes proteins upregulated while a negative ratio denotes proteins downregulated during the 2nd spawning season.

Table 2
Functional enrichment analysis of differentially abundant proteins in pikeperch seminal plasma collected during the first and second spawning seasons (up (↑) and down (↓) regulated in the second spawning season) according to Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genome (KEGG) pathway classification and Reactome (REAC) pathway analysis. Gene definitions are provided in Table 1.

Category	Term name	*P*-value	Genes
Biological processes			
GO:0006950	Response to stress	5.42e−7	↑*APOA2, PRDX2, SERPING1, HSPA8, AHCY, HSP90AA1, PARK7, HSP90AB1, SOD1, RAE1, FABP4, SKP1, PLD3, UCHL1, GOT1, BCL2;* ↓*SPARC, THBS4, F11, RNASE3, B2M, SERPINA10, GDF6, CHIA, AHSG, C1QTNF3, PROZ, FSTL1, APOE, MMP2, C9, C4B, LY75, PLG, CTSS, CFD;* ↑↓*C3*
GO:0002376	Immune system process	5.89e−6	↑*APOA2, PRDX2, SERPING1, TPD52, HSPA8, AHCY, HSP90AA1, PARK7, HSP90AB1, SOD1, SKP1, PLD3, BCL2;* ↓ *EEF1A1, THBS4, RNASE3, B2M, QPCT, CHIA, AHSG, ENPP2, CTSS, APOE, C9, C4B, LY75, PLG, CFD;* ↑↓*C3*
GO:0080134	Regulation of response to stress	3.23e−5	↑*PRDX2, SERPING1, HSPA8, HSP90AA1, PARK7, HSP90AB1, SOD1, RAE1, FABP4, SKP1, PLD3, BCL2;* ↓*F11, B2M, GDF6, AHSG, C1QTNF3, APOE, PLG;* ↑↓*C3*
GO:0006952	Defense response	5.81e−5	↑*APOA2, PRDX2, SERPING1, AHCY, PARK7, HSP90AB1, SOD1, FABP4, SKP1, PLD3, BCL2;* ↓*RNASE3, B2M, CHIA, AHSG, C1QTNF3, APOE, C9, C4B, LY75, CFD;* ↑↓*C3*
GO:0006954	Inflammatory response	3.24e−4	↑*APOA2, PRDX2, AHCY, PARK7, SOD1, FABP4, PLD3;* ↓*CHIA, AHSG, C1QTNF3, APOE, C4B, LY75, PROZ, SERPINA10;* ↑↓*C3*
GO:0048856	Developmental process	4.47e−4	↑*CKB, SLC26A3, RAE, PHGDH, HSP90AB1, SOD1, FABP4, BCL2, TPD52, VAPA, PLD3, UCHL1, SKP1, ARMC4, PRDX2;* ↓*CDH1, CDH23, GDF6, PLG, SPARC, MMP2, ENPP2, FURIN, FSTL1, B2M, C1QTNF6, AHSG, THBS4, UBE2L3;* ↑↓*C3*
GO:0051186	Cofactor metabolic process	6.28e−4	↑*MDH1, AHCY, PRDX2, PARK7, SOD1, GSTO1, GSTA1, GOT1, HSP90AA1, RAE1, CNDP2, DBI;* ↓*TCN2*
GO:0006508	Proteolysis	2.46e−3	↑*ITIH3, SERPING1, CNDP2, PARK7, HSP90AB1, SKP1, UCHL1;* ↓ *UBE2L3, F11, SERPINA10, AHSG, PROZ, FURIN, CTSS, APOE, MMP2 C4B, PLG, CFD;* ↑↓*C3*
GO:0003006	Developmental process involved in reproduction	3.10e−3	↑*SKP1, PARK7, SLC26A3, SOD1, BCL2, HSP90AB1;* ↓*C1QTNF6, GDF6*
GO:0098869	Cellular oxidant detoxification	4.56e−3	↑*GSTO1, PRDX2, GSTA1, PARK7, SOD1;* ↓*APOE*
GO:0030198	Extracellular matrix organization	3.46e−2	↓*PLG, CTSS, FBLN2, MMP2, CDH2, SPARC, FURIN, HMCN1*
Molecular Functions			
GO:0016209	Antioxidant activity	1.54e−4	↑*GSTO1, PRDX2, GSTA1, PARK7, SOD1;* ↓*APOE*
GO:0030414	Peptidase inhibitor activity	8.85e−4	↑*ITIH3, SERPING1;* ↓ *SERPINA10, AHSG, FURIN, C4B;* ↑↓*C3,*
GO:0030234	Enzyme regulator activity	8.99e3	↑*APOA2, ITIH3, SERPING1, HSP90AA1, PARK7, HSP90AB1;* ↓*UBE2L3, SERPINA10, AHSG, FURIN, APOE, C4B;* ↑↓ *C3*
GO:0004252	Serine-type endopeptidase activity	7.92e−3	↓*F11, PROZ, FURIN, MMP2, PLG, CFD*
GO:0004857	Enzyme inhibitor activity	1.21e−2	↑*APOA2, ITIH3, SERPING1;* ↓*SERPINA10, AHSG, FURIN, C4B;* ↑↓*C3*
GO:0008233	Peptidase activity	1.35e−2	↑*CNDP2, PARK7, UCHL1;* ↓*F11, PROZ, FURIN, CTSS, MMP2, PLG, CFD*
GO:0031625	Ubiquitin protein ligase binding	1.74e−2	↑*SKP1, HSPA8, HSP90AA1, HSP90AB1, CKB, UCHL1, BCL2;* ↓*UBE2L3,*
GO:0030060	L-malate dehydrogenase activity	0.31e−2	↑*MDH1, PHGDH*
GO:0005509	Calcium ion binding	3.57e−2	↑*TPD52;* ↓*PARC, THBS4, CDH2, PROZ, ENPP2, FBLN2, FSTL1, HMCN1, CDH23*
GO:0044183	Protein folding chaperone	4.19e−2	↑*HSPA8, HSP90AA1, HSP90AB1*
Cellular component			
GO:0005576	Extracellular region	8.41e−23	↑*APOA2, MDH1, GSTO1, ITIH3, PRDX2, SERPING1, HSPA8, CNDP2, AHCY, DBI, HSP90AA1, GSTA1, PARK7, HSP90AB1, SOD1, FABP4, CKB, PLD3, GOT1, PHGDH;* ↓*SPARC, EEF1A1, THBS4, F11, RNASE3, B2M, QPCT, SERPINA10, GDF6, CHIA, AHSG, C1QTNF3, PROZ, FURIN, ENPP2, TNFRSF6B, CTSSFSTL1, APOE, HMCN1, MMP2, C9, C4B, LY75, TCN2, PLG, CFD;* ↑↓*C3*
GO:0070062	Extracellular exosome	3.95e−18	↑*SOD1, CNDP2, SERPING1, DBI, ITIH3, MDH1, CKB, FABP4, PHGDH, HSP90AB1, HSP90AA1, HSPA8, AHCY, ARK7, PARK7, PRDX2, GST01, GSTA1, GOT1, APOA2;* ↓*LY75, F11, EEF1A1, PLD3, THBS4, C9, PLG, CFD, SERPINA10, FURIN, AHSG, FSTL1, PROZ, C4B, CQ10TNF3, B2M, APOE, QPCT, HMCN1;* ↑↓*C3*
GO:0101002	ficolin-1-rich granule	4.87e−8	↑*HSPA8, HSP90AA1, HSP90AB1;* ↓ *EEF1A1, QPCT, CTSS, CFD*
GO:0031012	Extracellular matrix	3.52e−7	↑*SERPING1;* ↓ *SPARC, THBS4, CDH2, AHSG, CTSS, FBLN2, APOE, HMCN1, MMP2, PLG*
GO:0012505	Endomembrane system	2.24e−3	↑*RAE1, TPD52, BCL2, PLD3, HSPA8, PARK7, SOD1, HSP90AA1, HSP90AB1, UCHL1, DBI, SERPING1, APOA2, ITIH3;* ↓*FURIN, SPARC, APOE, AHSG, B2M, THBS4, QPCT, PLG, PROZ, SERPINA10, EEF1A1, CTSS, FSTL1, RNASE3, CDH2, TCN2, CFD;* ↑↓*C3*
GO:0098805	Whole membrane	7.74e−3	↑*BCL2, PLD3, HSPA8, PARK7, HSP90AB1, VAPA;* ↓*SPARC, CDH2, FURIN, B2M, EEF1A1, APOE*
KEGG			
KEGG:04610	Complement and coagulation cascades	1.83e−5	↑*SERPING1;* ↓*F11, C9, C4B, PLG, CFD;* ↑↓*C3*
KEGG:04612	Antigen processing and presentation	2.12e−3	↑*HSPA8, HSP90AA1, HSP90AB1;* ↓*B2M, CTSS,*
KEGG:00270	Cysteine and methionine metabolism	8.21e−3	↑*MDH1, AHCY, GOT1, PHGDH*
REACTOME			
REAC:R-HSA-9613829	Chaperone Mediated Autophagy	2.01e−5	↑*HSPA8, HSP90AA1, PARK7, HSP90AB1;* ↓*EEF1A1*
REAC:R-HSA-8957275	Post-translational protein phosphorylation	2.81e−4	↑*APOA2;* ↓*SERPINA10, CDH2, AHSG, FSTL1, APOE;* ↑↓*C3*
REAC:R-HSA-168249	Innate Immune System	3.28e−3	↑*SERPING1, HSPA8, HSP90AA1, HSP90AB1, SKP1, PLD3, BCL2;* ↓*EEF1A1, RNASE3, B2M, QPCT, AHSG, CTSS, C9, C4B, CFD;* ↑↓*C3*



Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0006950	response to stress	32 of 3267	0.51	3.15e-08
GO:0050896	response to stimulus	48 of 7824	0.31	4.02e-08
GO:0002443	leukocyte mediated immunity	15 of 632	0.9	1.69e-07
GO:0016192	vesicle-mediated transport	22 of 1699	0.63	5.19e-07
GO:0006955	immune response	21 of 1560	0.65	6.34e-07
GO:0080134	regulation of response to stress	19 of 1299	0.69	1.13e-06
GO:0002252	immune effector process	16 of 927	0.76	2.40e-06
GO:0051186	cofactor metabolic process	12 of 467	0.93	3.06e-06
GO:0032101	regulation of response to external stimulus	16 of 955	0.74	3.10e-06
GO:0043312	neutrophil degranulation	12 of 485	0.91	3.98e-06

Fig. 4. Protein-protein interactions for the differentially expressed proteins of pikeperch seminal plasma identified by label-free liquid chromatography-mass spectrometry (LC–MS/MS). Different clusters of interacting proteins were identified using the STRING online platform. In the network, proteins are represented as nodes. Colors of the lines connecting the nodes represent different evidence types for protein linkage. The yellow halo indicates downregulated proteins, while the green halo indicates upregulated proteins during the 2nd spawning. Detailed information on protein names and abbreviations can be found in Table 1.

protein–protein interactions network consisted of 59 nodes and 146 edges. STRING analysis indicated the presence of two clusters. The majority of connected proteins were involved in the stress response, vesicle-mediated transport and immune response (Fig. 4).

Table 3
The ten major proteins identified in pikeperch seminal plasma after liquid chromatography with tandem mass spectrometry (LC–MS/MS) based on total spectra counts from both spawning seasons.

No	Accession number	Protein name	Total spectra count
1	XP_031139526.1	apolipoprotein A-I-like	622
2	XP_031150158.1	transferrin-a isoform X1	492
3	XP_031135425.1	type-4 ice-structuring protein LS-12-like	281
4	XP_031145028.1	complement C3-like	280
5	XP_031147766.1	hemopexin isoform X1	247
6	XP_031151286.1	alpha 1-antitrypsin homolog	183
7	XP_035857333.1	cadherin-23-like	157
8	XP_035862199.1	apolipoprotein A-II	162
9	XP_035852865.1	papilin	146
10	XP_031140202.1	hyaluronan-binding protein 2	105

Comprehensive functional annotation of seminal plasma proteins identified in pikeperch from both spawning seasons

Ten major pikeperch seminal plasma proteins based on spectral counts were obtained (Table 3). To obtain an overview of the biological associations of the pikeperch seminal plasma proteins out of 850 identified proteins, 789 were submitted to g:Profiler analysis (*Homo sapiens* was selected as target organism) because 29 proteins were identified as uncharacterized/hypothetical with no similarity to mammals, and 16 proteins were not found in humans, including ice-structuring proteins, saxitoxin and tetrodotoxin-binding proteins, hibernation-specific plasma proteins, and lectins (galactose-specific lectin nattectins, fucolectins, L-rhamnose binding lectin, natterin). As a result, 805 proteins were annotated to a GO database and classified into 88 functional groups (Supplementary Material S2). The top 10 significant terms for molecular functions, biological processes, and cellular components are presented in Fig. 5. The top-ranked biological processes were vesicle-mediated transport, response to stress, exocytosis, immune system process, catabolic process, transport, small molecule metabolic process, proteolysis, exocytosis, oxoacid metabolic process, and

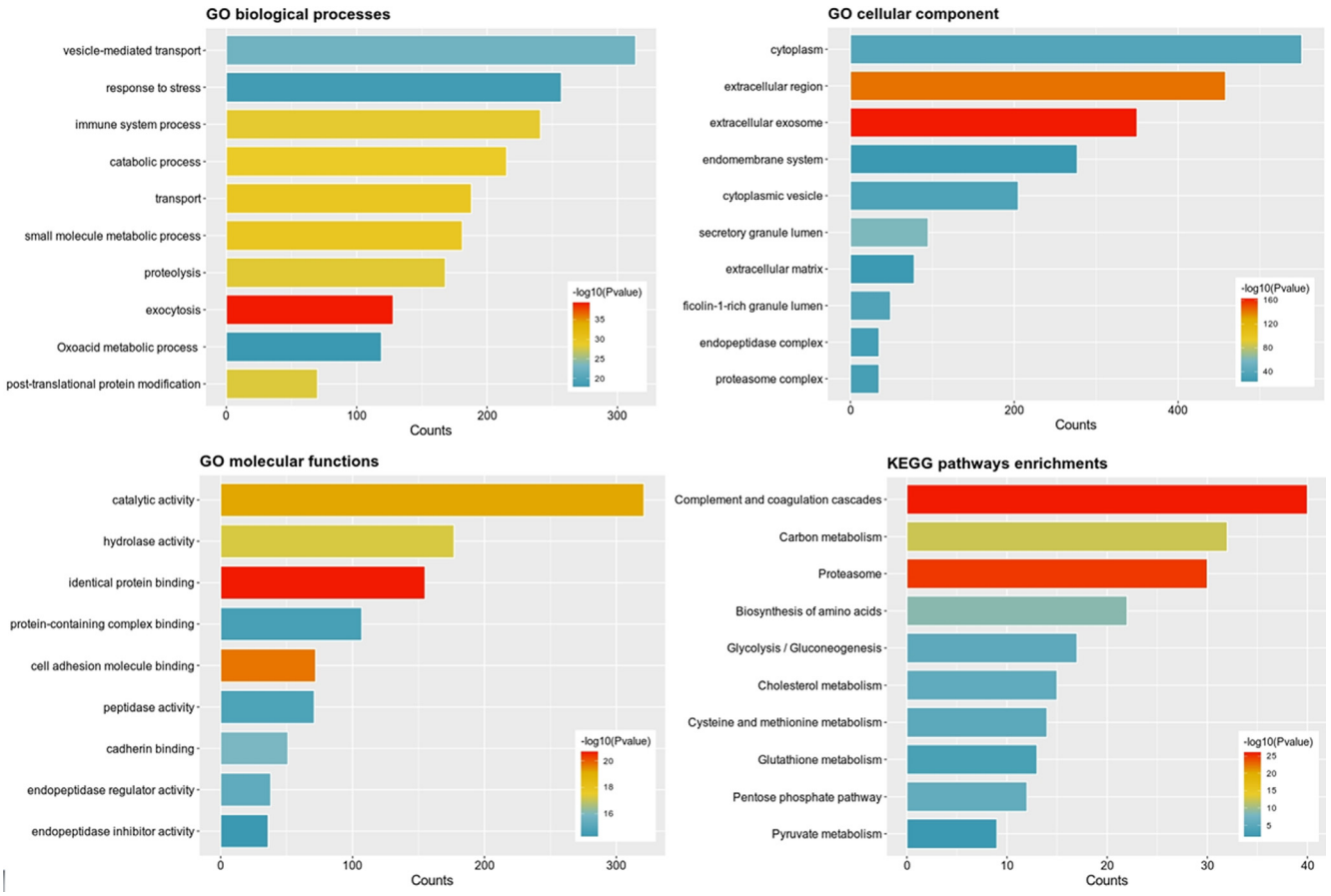


Fig. 5. Gene Ontology (GO) analysis of biological process, molecular function, cellular component and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification of pikeperch seminal plasma proteins.

post-translational protein modification. The seminal plasma proteins were involved in catalytic activity, hydrolase activity, identical protein binding, protein-containing complex binding, cell adhesion molecule binding, peptidase activity, cadherin binding, endopeptidase regulator activity, and endopeptidase inhibitor activity. The top-ranked KEGG pathways were complement and coagulation cascade, proteasome, and metabolic pathways including glycolysis, pentose phosphate pathway, lipid and amino acid metabolism.

An additional search of seminal plasma proteins using the UniProt GO annotation database revealed their involvement in reproductive processes such as developmental processes involved in reproduction, reproductive system development, gamete generation, gonadal development, binding of sperm to the zona pellucida, male gamete generation, spermatogenesis, fertilization and response to hormones (Supplementary Table S1).

Discussion

This work represents the first in-depth proteomic characterization of pikeperch seminal plasma and reveals differences in semen quality and quantity parameters and seminal plasma protein profiles between the first and second spawning seasons of pikeperch males kept in captivity. Using mass spectrometry-based label-free quantitative proteomics, we identified 850 proteins in the seminal plasma of pikeperch from both spawning seasons, and 65 proteins were found to be differentially abundant between the seminal plasma of the first and second spawning seasons. The majority of DAPs were involved in stress and immune response, developmental processes, cofactor metabolic process, proteolysis, cellular oxidant detoxification and organization of the extracellular matrix. In addition, several proteins unique to pikeperch seminal plasma were identified.

Sperm quality and quantity parameters over two spawning seasons

Our results demonstrated that hormonal stimulation was successful for both seasons and that semen with a high percentage of motile spermatozoa was produced. However, we observed much lower sperm concentrations and volumes in mature 3+-year-old Eurasian pikeperch males spawned for the first time compared to males spawned for the second season, which likely results from incomplete gonadal maturation. This result is in accordance with the results of Khendek et al. (2018), who demonstrated that farmed pikeperch males reproduced for the first time exhibited lower reproductive performance, with only 40% spermatozoa (in large part comprising spermatocytes, spermatids and spermatogonia) compared to males matured in the 2nd or higher maturation episode. The poor quality of spermatogenesis in the first year of sexual activity was also demonstrated for yellow perch (Ciereszko et al., 1998). In our study, in the second spawning, spermatogenesis was clearly more efficient, leading to the production of a higher number of spermatozoa and consequently a higher sperm concentration that was within the range reported for wild pikeperch semen (Nynca et al., 2010; Sarosiek et al., 2016). The higher sperm volume and total number of collected semen as well as the higher protein concentration of seminal plasma collected during the second spawning indicate an advanced gonadal maturation process.

On the other hand, in the second spawning, we observed a slight drop in seminal plasma osmolality, from 294 (1st spawning; this value was within the range previously reported for wild pikeperch [289–342 mOsm/kg], Sarosiek et al., 2016) to 278 mOsm/kg (2nd spawning), which may indicate disturbances to osmotic balance for this period.

Differentially abundant proteins involved in stress and immune responses

Our results indicated that changes in seminal plasma composition between the first and second spawning seasons are attributed to stress and immune responses. The majority of innate immunity components were downregulated during the 2nd spawning, including RNASE3, TNFRSF6B, AHSG, components of the coagulation process (coagulation factor F11, plasminogen, protein Z and protein Z inhibitor [SERPINA10]) and complement components (C3, C4A, C9, complement factor D), the latter of which agree with the increase in SERPING1, a negative regulator of the complement cascade. The downregulation of innate immune-related proteins agrees with suppressive effect of chronic stress on the immune response in fish (Douxfils et al., 2012; Uren Webster et al., 2018). At the same time, proteins of adaptive immunity, including antigen processing and presentation, were both downregulated (B2M, CTSS, LY75) and upregulated (HSP90AB1, HSP90AA1, HSPA8B, HSPA8). On the other hand, proteins involved in the cellular response to oxidative stress (SOD1, GSTO1, GSTA1, PRDX2, PARK7) and stress proteins (HSPs, UHCL1, Douxfils et al., 2011) were enriched at the 2nd spawning. It is well known that acute and chronic stress may induce quite different effects on proteome and immune response, and it is possible that both types of stress were present in fish of 2nd spawning. Induction of antioxidants and HSPs is a characteristic feature of the cellular response to the acute stress, however, similarly to our study, elevation of stress proteins was observed in response to chronic stress in fish (Douxfils et al., 2011 and 2012; Uren Webster et al., 2018). Moreover, increase in these proteins suggests that males during second spawning displayed a higher sensitivity to the acute stressors after exposure to chronic stress which supports the modulation of the acute response by previous chronic stress in Eurasian perch (Douxfils et al., 2011). Our results provide important information to better understand the relationship between chronic and acute stress and the immune response of pikeperch maintained in captivity. This also confirms that proteins contained in seminal plasma are of high relevance as markers of immune and stress status in fishes (Dietrich et al., 2014).

To our knowledge, we identified lymphocyte antigen 75 (Ly75) in fish seminal plasma for the first time. This protein is recognized as a spermatogonia A cell surface marker in fish (Yang et al., 2018). Its higher abundance during the first spawning suggests incomplete maturation of the testis, as indicated by sperm characteristic analysis. As such, Ly75 can be a potential marker for evaluating the maturation of the reproductive system in male pikeperch.

We identified saxitoxin and tetrodotoxin-binding protein 1, which were upregulated during the second spawning. Moreover, we found that the uncharacterized protein LOC116056820 showed 90% sequence coverage with saxitoxin and tetrodotoxin-binding protein 1 from *Larimichthys crocea*, which was downregulated during this spawning. Both proteins are capable of binding to neurotoxins produced by bacteria: saxitoxin and tetrodotoxin, which block voltage-gated sodium channels. To date, saxitoxin and tetrodotoxin-binding proteins have been identified in several species of pufferfish with roles in the transport and accumulation and/or excretion of saxitoxin and tetrodotoxin, constituting a toxin-tolerant system in these fish (Yotsu-Yamashita et al., 2018). Recently, their role in reducing the toxicity of tributylin, a strong endocrine disruptor, was suggested (Takai et al., 2020). The identification of saxitoxin and tetrodotoxin-binding protein 1 in the seminal plasma of pikeperch suggests its role in detoxification mechanisms that are important in protecting reproductive tract tissue and spermatozoa against xenobiotic toxicity. Further study of the specific role of this protein in the fish reproductive system with a special focus on the identification of target xenobiotics is advised.

Differentially abundant proteins involved in developmental processes

The majority of DAPs (17 up- and 18 downregulated during the second spawning) were connected to the GO term “developmental process”. Most importantly, we found 11 proteins associated with developmental processes involved in reproduction, especially testis development and spermatogenesis. The identification of UCHL1, UBE2L3 and SKP1 (components of ubiquitin E3 ligase) is consistent with the importance of ubiquitination-mediated proteolysis at different stages of spermatogenesis and the control of sperm quality. SKP1 is recognized as a key protein in the production of mature spermatozoa (Guan et al., 2012), which agrees with higher expression during the 2nd spawning. We also identified several proteins upregulated in the 2nd spawning that are involved in different processes related to sperm production in vertebrates. HSP90AB1, in addition to its role in protein folding during the stress response, also participates in orchestrating both the morphological differentiation of germ cells during spermatogenesis and their post-testicular maturation (Dun et al., 2012). ARMC4 is required for spermatid maturation, including differentiation into elongated shapes and separation from each other to form spermatozoa (Cheng et al., 2013). TPD52 is implicated in testis development and spermatogenesis, while Bcl2l13 (bcl-rambo) controls germ cell apoptosis during spermatogenesis (Nakazawa et al., 2016). Upregulation of SOD1, GSTO1, GSTA1, PARK7 and PRDX2 can be part of the mechanism controlling the level of reactive oxygen species (**ROS**) generation at a low-to-moderate range, which is beneficial or even indispensable in cellular proliferation, differentiation, and survival. Moreover, these proteins can be important for the protection of spermatozoa against oxidative stress during post-testicular storage. Solute carrier 26, a transmembrane protein, acts as a chloride/bicarbonate exchanger across the plasma membrane of spermatozoa and may contribute to the composition and pH of secreted fluids (Hihnala et al., 2006). It is likely that in fish, this protein is linked to the final step of sperm maturation because buffers containing sodium bicarbonate and elevated pH have been found to be useful for the *in vitro* maturation of testicular semen (Morisawa and Morisawa, 1988). In summary, our results contribute to a better understanding of spermatogenesis and post-testicular maturation of spermatozoa, including the regulation of ROS generation, bicarbonate production, sperm elongation and separation.

Among proteins upregulated during the 1st spawning, we identified GSDF, a unique member of the transforming growth factor β superfamily, found only in teleosts (Sawatari et al., 2007). In mature rainbow trout, GSDF is related to early stages of spermatogenesis and was predominantly detected in Sertoli cells and spermatogonia, indicating its role in the proliferation of spermatogonia (Sawatari et al., 2007). Moreover, GSDF is recognized as a marker of the male gonad, showing high expression in males during sex differentiation and decreasing expression in mature testes (Zhu et al., 2018). We also identified EEF1A1, an indispensable factor in protein synthesis expressed in spermatogonia, and CTRP3, which, in addition to its anti-inflammatory role in inflammation, is linked with cellular proliferation, growth and development. In summary, proteins upregulated at first spawning seem to be related to the early stage of spermatogenesis and testis development in male

pikeperch, whereas proteins upregulated at the 2nd spawning are associated with the late phase of spermatogenesis and sperm maturation.

Differentially abundant proteins involved in extracellular matrix organization

The seminal plasma of the 1st spawning was characterized by a higher abundance of proteins involved in tissue remodeling, including MMP2, which plays a crucial role in extracellular matrix (**ECM**) degradation, and secreted non-structural matricellular glycoproteins (SPARC, THBS4 and fibulins: FBLN2 and HMCN1) that regulate ECM assembly and cell–matrix interactions. This observation is in agreement with the dynamic remodeling of ECM components, which occurs in accordance with morphological testis alteration throughout the fish reproductive cycle (Santana and Quagio-Grassiotto, 2014). The ECM plays a significant role in regulating spermatogenesis, particularly regarding spermatogonia and Sertoli cells, since these cells are in physical contact with the basement membrane, a modified form of ECM. In fish, MMP2 is a main enzyme involved in the degradation of many ECM compounds during tissue degradation and remodeling and can also regulate cell–cell and cell-matrix signaling. Furthermore, in mammalian testis, MMP2 was shown to be activated when germ cells, especially spermatids, were detached from the epithelium (Siu and Cheng, 2008). Its activity is tightly regulated, as identified in our study by SPARC, FURIN, FSTL1 and the plasmin/plasminogen system. We also found higher amounts of CDH2 and CDH23, transmembrane/membrane proteins that mediate cell–cell adhesion, especially to maintain junctions between Sertoli cells and spermatogonia or primary spermatocytes during spermatogenesis. In summary, our results suggest that extensive modeling of the ECM, which is essential in supporting Sertoli and germ cell functions, is especially important during the first spawning season.

In our study, we assumed that observed differences reflect changes in maturation stage between first- and second-time spawners. However, we are also aware that some environmental factors could influence our results in a view of not exactly the same conditions in two different years. As such modulation effect of environmental factors should be taken into consideration and studied in more detail in the future studies.

Seminal plasma proteome and its functions

LC–MS analysis yielded the first described proteomic data for pikeperch seminal plasma and, to our knowledge, the largest seminal plasma proteome (850 proteins) reported for teleost fish. To date, 152 and 186 proteins have been mapped in the seminal plasma of rainbow trout and carp, respectively, using 1D SDS–PAGE combined with LC–MS (Nynca et al., 2014; Dietrich et al., 2014), while 345 proteins have been identified in Chinook salmon seminal plasma using in-solution digestion coupled with LC–MS (Gombar et al., 2017). Taking advantage of the large seminal plasma proteome, we were able to perform comprehensive bioinformatic analysis and indicate the major functions of pikeperch seminal plasma proteins.

Importantly, the most enriched function of pikeperch seminal plasma proteins was vesicle-mediated transport with a substantial number of proteins associated with exosomes. Seminal plasma proteins associated with exosomes were also identified in salmon seminal plasma (Gombar et al., 2017), and recently, exosomes were isolated from sole (*Cynoglossus semilaevis*) seminal plasma (Zhang et al., 2020). These results together with our data strongly suggest that exosome-mediated events are involved in spermatogenesis and sperm maturation in fish. Sperm-exosome fusion is essential for the transfer of proteins to transcriptionally and translationally silent spermatozoa during post-testicular maturation (Sullivan et al., 2005). Exosome-associated proteins are involved in energy pathways, protein metabolism, signaling, antioxidant systems, and protection against antimicrobial and proteolytic attraction (Sullivan et al., 2005). Identified in the present study, proteins included exosome-marker proteins, such as PDCD6IP, YWHAE, ADAM10, FLOT1, RAB7A and LAMP2, which can be used as biomarkers for further studies of the mechanism of exosome participation in spermatogenesis and sperm maturation in fish.

The other most enriched biological processes included stress and immune response, catabolic process, transport, metabolism (glycolysis/gluconeogenesis, pentose phosphate pathway, amino acid metabolism), proteolysis, exocytosis and post-translational protein modifications, which correspond to the major role of fish seminal plasma in the protection of spermatozoa against oxidative stress and microbial attraction, energy supply, maintenance of sperm membrane composition and temporal regulation of these processes by proteinase/inhibitors. This is in agreement with previously published results (Dietrich et al., 2014; Nynca et al., 2014; Gombar et al., 2017). In comparison to earlier results, we provided a much higher number of proteins and extended our knowledge of particular proteins participating in specific functions (for example, immune response and reproduction, Supplementary Material S1, Supplementary Table S1 and Supplementary Table S2), which is very important for better understanding the mechanism underlying the male reproductive system in pikeperch as well as for the identification of novel biomarkers for sperm quality.

Major pikeperch seminal plasma proteins

In our study, we identified major proteins (including acute phase response proteins such as apolipoprotein A1, transferrin, complement C3, hemopexin, alpha1-antitrypsin, and apolipoprotein A2) in domesticated pikeperch seminal plasma. These proteins were previously identified in other fish species, such as carp and rainbow trout (Dietrich et al., 2014; Nynca et al., 2014), suggesting that the general protective mechanism in teleosts is significantly exerted by acute phase proteins, which are well conserved in fish seminal plasma. On the other hand, some major proteins do appear to be specific for pikeperch seminal plasma (e.g., type-4-ice-structuring protein LS-12-like, papilin and hyaluronian binding protein 2), which suggests that the fish seminal plasma composition described here can be specific for the percid family. The relationship of such proteins to the specificity of reproduction of percid fish must be investigated in future studies.

Antifreeze proteins in pikeperch seminal plasma

An important finding of our study was the identification of antifreeze proteins, such as type-4 ice-structuring protein LS-12-like, classified as a major seminal protein of pikeperch, and type-2 ice-structuring protein, which was present with lower abundance. To our knowledge, this is the first report providing direct evidence for the presence of antifreeze proteins in fish semen. Their presence has been postulated in the seminal plasma of wolfish using indirect methods based on changes in freezing points (Le François et al., 2008). The main function of antifreeze proteins is to confer protection to cells by decreasing the freezing point, modifying the ice crystal formation process, preventing recrystallization and stabilization of the cell membrane via interaction with phospholipids. Antifreeze activity is mainly related to antifreeze glycoprotein and types I-III antifreeze proteins. The structure of a lipoprotein-like antifreeze protein (type IV) identified in our study as a major protein is completely unrelated to the structure of other antifreeze proteins present in fish and exhibits *in vitro* antifreeze properties; however, its in vivo function is still unclear. Its role in

lipid metabolism was suggested in Nile tilapia (Ammar et al., 2018). The biological role of fish antifreeze proteins is mainly related to protection against cold-induced damage to the membrane (for example, polar fish) at extremely low temperatures. Surprisingly, antifreeze activity was also demonstrated in temperate fish; however, its function remains unknown. Percid fish living in temperate zones require an extended winter period of 3–5 months with temperatures below 10 °C for pikeperch (Żarski et al. 2019) or below 6 °C for perch (Fontaine et al., 2015) for proper gonadal development and spermiation. It is therefore possible that antifreeze proteins have a role in gamete protection during this vernalization period.

Hibernation-specific proteins in pikeperch seminal plasma

We also identified the hibernation-specific plasma protein HP-55-like in pikeperch seminal plasma. In mammals, HP-55 (a homolog to α 1-antitrypsin) together with the HP20c complex (which is formed from three structurally homologous proteins: HP20, 25 and 27) forms a 140-kDa hibernation-specific protein complex. Hibernation-specific protein genes were also found in non-hibernating mammals, and it was suggested that hibernation protein-regulated liver–brain circuits may couple seasonal changes in the environment to alterations in physiology (Seldin et al., 2014). Pikeperch, like other fish, does not undergo hibernation; for this species, decreasing temperatures activate the hypothalamic–pituitary–gonadal axis, and nutrients are mobilized and directed to the maturing gonads, which is an energy-demanding process (Fontaine et al., 2015). This is the first report concerning the existence of hibernation-specific proteins in fish, and their function in pikeperch seminal plasma is not related to hibernation but may be associated with an adaptive mechanism to cold temperature, as indicated by the presence of identified antifreeze proteins (see above). It is also possible that HP−55 is related to photoperiod regulation of fish reproduction in view of the results of Seldin et al. (2014) showing seasonal oscillation of the hibernation protein complex in non-hibernating mammals.

Vitellogenin in pikeperch seminal plasma

Surprisingly, we identified vitellogenins (VTG1-3) in the seminal plasma of two males from the first spawning; these proteins were undetected during the second spawning (Supplementary Material S1). Vitellogenin is a precursor of egg yolk proteins; its synthesis is controlled by estradiol-17β in the liver of female fish during maturation. In males, the vitellogenin gene is silent but can be activated after exposure to exogenous estrogen, and the presence of vitellogenin in the blood of males has been widely used as a biomarker of exposure to estrogenic endocrine-disrupting chemicals present in the environment or food (Mills et al., 2003). Moreover, estrogen exposure induced extrahepatic expression and synthesis of vitellogenin in different fish tissues, including testes (Kobayashi et al., 2005). It seems that stress is rather not responsible for vitellogenin production in males because it was demonstrated that stress induces masculinization process in fish (Nozu and Nakamura, 2015). The presence of vitellogenin in pikeperch seminal plasma may reflect endocrine disruption during testis development in immature males at first spawning.

Conclusion

The identification of a large number of seminal plasma proteins provides a valuable resource for understanding the functions of seminal plasma and the molecular mechanism of testicular development and spermatogenesis at the protein level. For the first time, important proteins not described previously in fish seminal plasma were identified, including antifreeze proteins, hibernation-specific plasma proteins, lectins and vitellogenin. In addition, the detection of proteins associated with exosomes shed light on their involvement in spermatogenesis and sperm maturation. Males spawned for the first time were characterized by incompletely mature gonads and the expression of proteins associated with the early phase of spermatogenesis and ECM organization. On the other hand, males spawned for the second time exhibited advanced gonadal maturation and expression of proteins related to the late stage of spermatogenesis and sperm maturation, including regulation of ROS generation, bicarbonate production, sperm elongation and separation. The identified proteins may serve as potential biomarkers of testis maturation and sperm quality in pikeperch. The possible modulation effect of environmental factors should be tested in the future studies.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100279.

Ethics approval

Not applicable.

Data and model availability statement

None of the data were deposited in an official repository. Proteomic raw data are available upon request from the corresponding author.

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Declaration of interest

None.

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References

Ammar, A.Y., El Nahas, A.F., Mahmoud, S., Barakat, M.E., Hassan, A.M., 2018. Characterization of type IV antifreeze gene in Nile tilapia (*Oreochromis niloticus*) and influence of cold and hot weather on its expression and some immune-related genes. Fish Physiology and Biochemistry 44, 515–525.

Billard, R., 1983. Effects of coelomic and seminal fluids and various saline diluents on the fertilizing ability of spermatozoa in the rainbow trout, *Salmo gairdneri*. Journal of Reproduction and Fertility 68, 77–84.

Blecha, M., Kristan, J., Samarin, A.M., Rodina, M., Policar, T., 2015. Quality and quantity of pikeperch (*Sander lucioperca*) spermatozoa after varying cold water treatments. Journal of Applied Ichthyology 31, 75–78.

Cheng, W., Ip, Y.T., Xu, Z., 2013. Gudu, an Armadillo repeat-containing protein, is required for spermatogenesis in Drosophila. Gene 531, 294–300.

Ciereszko, R.E., Dabrowski, K., Ciereszko, A., Ottobre, J.S., 1998. Plasma concentrations of steroid hormones in male yellow perch, *Perca flavescens*: the effect of age and photothermal manipulation. Environmental Biology of Fishes 51, 97–105.

Dietrich, M.A., Arnold, G.J., Nynca, J., Fröhlich, T., Otte, K., Ciereszko, A., 2014. Characterization of carp seminal plasma proteome in relation to blood plasma. Journal of Proteomics 98, 218–232.

Douxfils, J., Mandiki, S.N., Marotte, G., Wang, N., Silvestre, F., Milla, S., Henrotte, E., Vandecan, M., Rougeot, C., Mélard, C., Kestemont, P., 2011. Does domestication process affect stress response in juvenile Eurasian perch Perca fluviatilis?. Comparative Biochemistry and Physiology Part - A: Molecular and Integrative Physiology 159, 92–99.

Douxfils, J., Deprez, M., Mandiki, S.N., Milla, S., Henrotte, E., Mathieu, C., Silvestre, F., Vandecan, M., Rougeot, C., Mélard, C., Dieu, M., Raes, M., Kestemont, P., 2012. Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication–clues to physiological acclimation and humoral immune modulations. Fish and Shellfish Immunology 33, 1112–1122.

Dun, M.D., Aitken, R.J., Nixon, B., 2012. The role of molecular chaperones in spermatogenesis and the post-testicular maturation of mammalian spermatozoa. Human Reproduction Update 18, 420–435.

Elias, J.E., Haas, W., Faherty, B.K., Gygi, S.P., 2005. Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. Nature Methods 2, 667–675.

Fontaine, P., Wang, N., Hermelink, B., 2015. Broodstock management and control of the reproduction cycle. In: Kestemont, P., Dabrowski, K., Summerfelt, R.C. (Eds.), Biology and culture of percid fishes—principles and practices. Springer, New York, NY, USA, pp. 103–122.

Gombar, R., Pitcher, T.E., Lewis, J.A., Auld, J., Vacratsis, P.O., 2017. Proteomic characterization of seminal plasma from alternative reproductive tactics of Chinook salmon (*Oncorhynchus tswatchysha*). Journal of Proteomics 157, 1–9.

Guan, Y., Leu, N.A., Ma, J., Chmátal, L., Ruthel, G., Bloom, J.C., Lampson, M.A., Schimenti, J.C., Luo, M., Wang, P.J., 2012. SKP1 drives the prophase I to metaphase I transition during male meiosis. Science. Advances 6, eaaz2129. https://doi.org/10.1126/sciadv.aaz2129.

Hihnala, S., Kujala, M., Toppari, J., Kere, J., Holmberg, C., Höglund, P., 2006. Expression of SLC26A3, CFTR and NHE3 in the human male reproductive tract: role in male subfertility caused by congenital chloride diarrhoea. Molecular Human Reproduction 12, 107–111.

Judycka, S., Dietrich, M.A., Żarski, D., Karol, H., Palińska-Żarska, K., Błażejewski, M., Ciereszko, A., 2021. Towards standardization of the cryopreservation procedure of cultured pikeperch (*Sander lucioperca*) semen. Aquaculture 538, 736539.

Khendek, A., Chakraborty, A., Roche, J., Ledoré, Y., Personne, A., Policar, T., Żarski, D., Mandiki, R., Kestemont, P., Milla, S., Fontaine, P., 2018. Rearing conditions and life history influence the progress of gametogenesis and reproduction performances in pikeperch males and females. Animal 12, 2335–2346.

Kobayashi, K., Tamotsu, S., Yasuda, K., Oishi, T., 2005. Vitellogenin-immunohistochemistry in the liver and the testis of the medaka, *Oryzias latipes*, exposed to 17beta-estradiol and p-nonylphenol. Zoological Science 22, 453–461.

Le François, N.R., Lamarre, S.G., Tveiten, H., Blier, P.U., Bailey, J., 2008. Sperm cryoconservation in *Anarhichas* sp., endangered cold-water aquaculture species with internal fertilization. Aquaculture International 16, 273–279.

Malinowska, A., Kistowski, M., Bakun, M., Rubel, T., Tkaczyk, M., Mierzejewska, J., Dadlez, M., 2012. Diffprot - software for non-parametric statistical analysis of differential proteomics data. Journal of Proteomics 75, 4062–4073.

Mills, L.J., Gutjahr-Gobell, R.E., Horowitz, D.B., Denslow, N.D., Chow, M.C., Zaroogian, G.E., 2003. Relationship between reproductive success and male plasma vitellogenin concentrations in cunner, *Tautogolabrus adspersus*. Environmental Health Perspectives 111, 93–100.

Morisawa, S., Morisawa, M., 1988. Induction of potential for sperm motility by bicarbonate and pH in rainbow trout and chum salmon. Journal of Experimental Biology 136, 13–22.

Nakazawa, M., Matsubara, H., Matsushita, Y., Watanabe, M., Vo, N., Yoshida, H., Yamaguchi, M., Kataoka, T., 2016. The human Bcl-2 family member Bcl-rambo localizes to mitochondria and induces apoptosis and morphological aberrations in Drosophila. PLoS One 11,. https://doi.org/10.1371/journal.pone.0157823 e0157823.

Nozu, R., Nakamura, M., 2015. Cortisol administration induces sex change from ovary to testis in the protogynous Wrasse, *Halichoeres trimaculatus*. Sexual Development 9, 118–124.

Nynca, J., Arnold, G.J., Fröhlich, T., Otte, K., Flenkenthaler, F., Ciereszko, A., 2014. Proteomic identification of rainbow trout seminal plasma proteins. Proteomics 14, 133–140.

Nynca, J., Horváth, A., Dietrich, M., Müller, T., Karol, H., Urbányi, B., Kotrik, L., Ciereszko, A., 2010. Serine proteinase inhibitors in the seminal plasma of percid fish. Journal of Applied Ichthyology 26, 742–745.

Policar, T., Schaefer, F.J., Panana, E., Meyer, S., Teerlinck, S., Toner, D., Żarski, D., 2019. Recent progress in European percid fish culture production technology—tackling bottlenecks. Aquaculture International 27, 1151–1174.

Santana, J.C., Quagio-Grassiotto, I., 2014. Extracellular matrix remodeling of the testes through the male reproductive cycle in Teleostei fish. Fish Physiology and Biochemistry 40, 1863–1875.

Sarameh, S.P., Falahatkar, B., Takami, G.A., Efatpanah, I., 2012. Effects of different photoperiods and handling stress on spawning and reproductive performance of pikeperch *Sander lucioperca*. Animal Reproduction Science 132, 213–222.

Sarosiek, B., Dryl, K., Krejszeff, S., Żarski, D., 2016. Characterization of pikeperch (*Sander lucioperca*) milt collected with a syringe and a catheter. Aquaculture 450, 14–16.

Sawatari, E., Shikina, S., Takeuchi, T., Yoshizaki, G., 2007. A novel transforming growth factor-beta superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). Developmental Biology 301, 266–275.

Seldin, M.M., Byerly, M.S., Petersen, P.S., Swanson, R., Balkema-Buschmann, A., Groschup, M.H., Wong, G.W., 2014. Seasonal oscillation of liver-derived hibernation protein complex in the central nervous system of non-hibernating mammals. Journal of Experimental Biology 217, 2667–2679.

Siu, M.K., Cheng, C.Y., 2008. Extracellular matrix and its role in spermatogenesis. Advances in Experimental Medicine and Biology 636, 74–91.

Sullivan, R., Saez, F., Girouard, J., Frenette, G., 2005. Role of exosomes in sperm maturation during the transit along the male reproductive tract. Blood Cells, Molecules and Diseases 35, 1–10.

Takai, Y., Mizoguchi, N., Kinoshita, M., Qiu, X., Shimasaki, Y., Oshima, Y., 2020. Establishment of a Japanese medaka *(Oryzias latipes*) transgenic line expressing *Takifugu rubripes* pufferfish saxitoxin and tetrodotoxin binding protein 1, and evaluation of tributyltin toxicity via in ovo nanoinjection. Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology 234,. https://doi.org/10.1016/j.cbpc.2020.108785 108785.

Teletchea, F., Fontaine, P., 2014. Levels of domestication in fish: implications for the sustainable future of aquaculture. Fish and Fisheries 15, 181–195.

Uren Webster, T.M., Rodriguez-Barreto, D., Martin, S.A.M., Van Oosterhout, C., Orozco-terWengel, P., Cable, J., Hamilton, A., Garcia De Leaniz, C., Consuegra, S., 2018. Contrasting effects of acute and chronic stress on the transcriptome, epigenome, and immune response of Atlantic salmon. Epigenetics 13, 1191–1207.

Yang, Y., Liu, Q., Ma, D., Song, Z., Li, J., 2018. A potential germ cell-specific marker in Japanese flounder, *Paralichthys olivaceus*: identification and characterization of lymphocyte antigen 75 (Ly75/CD205). Journal of Oceanology and Limnology 36, 1342–1348.

Yotsu-Yamashita, M., Nagaoka, Y., Muramoto, K., Cho, Y., Konoki, K., 2018. Pufferfish saxitoxin and tetrodotoxin binding protein (PSTBP) analogues in the blood plasma of the pufferfish *Arothron nigropunctatus*, *A. hispidus, A. manilensis, and Chelonodon patoca*. Marine Drugs 16, 224. https://doi.org/10.3390/md16070224.

Żarski, D., Ben Ammar, I., Bernáth, G., Baekelandt, S., Bokor, Z., Palińska-Żarska, K., Fontaine, P., Horváth, Á., Kestemont, P., Mandiki, S.N.M., 2020. Repeated hormonal induction of spermiation affects the stress but not the immune response in pikeperch (*Sander lucioperca*). Fish and Shellfish Immunology 101, 143–151.

Żarski, D., Fontaine, P., Roche, J., Alix, M., Blecha, M., Broquard, C., Król, J., Milla, S., 2019. Time of response to hormonal treatment but not the type of a spawning agent affects the reproductive effectiveness in domesticated pikeperch, *Sander lucioperca*. Aquaculture 503, 527–536.

Zhang, B., Zhao, N., Jia, L., Che, J., He, X., Liu, K., Bao, B., 2020. Identification and application of piwi-interacting RNAs from seminal plasma exosomes in *Cynoglossus semilaevis*. BMC Genomics 21, 302. https://doi.org/10.1186/s12864-020-6660-7.

Zhu, Y., Meng, L., Xu, W., Cui, Z., Zhang, N., Guo, H., Wang, N., Shao, C., Chen, S., 2018. The autosomal Gsdf gene plays a role in male gonad development in Chinese tongue sole (*Cynoglossus semilaevis*). Scientific Reports 8, 17716. https://doi.org/10.1038/s41598-018-35553-7.