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Proteomic analysis of carp seminal plasma provides insights into the immune response to bacterial infection of the male reproductive system

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ABSTRACT

Aeromonas salmonicida is recognized as a significant bacterial pathogen in ulcerative disease of cyprinid fish. However, the mechanism of immunity to these bacteria in common carp is still not well understood, especially the immune regulation in the gonad to bacterial infection. The aims of our study were to analyze changes in the seminal plasma proteome following A. salmonicida infection in carp males. The observed pathological changes in the tissue (liver, spleen, kidney and testis) morphology and upregulation of immune-related genes (tnfa2, il6a) confirmed the successful infection challenge. Using mass spectrometry-based label-free quantitative proteomics, we identified 1402 seminal plasma proteins, and 44 proteins (20 up- and 24 downregulated) were found to be differentially abundant between infected and control males. Most differentially abundant proteins were involved in the immune response mechanisms, such as acute phase response, complement activation and coagulation, inflammation, lipid metabolism, cell-cell and cell-matrix adhesion, creatine-phosphate biosynthesis and germ cell-Sertoli cell junction signaling. Bacterial infection also caused profound changes in expression of selected genes in the testis and hematopoietic organs, which contributed to changes in seminal proteins. The altered seminal proteins and bacterial proteins in seminal plasma may serve as valuable markers of infection in the testis.

1. Introduction

Aquaculture is affected primarily by microbial pathogens mostly of bacterial origin [1], wherein Aeromonas salmonicida is widespread in aquatic environments and is considered the etiological factor of many fish diseases [2]. A. salmonicida is a gram-negative bacterium that causes furunculosis, a systemic disease that affects salmonids; however, the high mortality and morbidity caused by this bacterium was subsequently found in nonsalmonid species, such as catfish, tilapia, eels, turbot, halibut and Cyprinids, causing global economic losses [3]. Nevertheless, very little was reported about A. salmonicida infection in Cyprinids.

The common carp (Cyprinus carpio), which is the research object in our study, is commercially important fish species of significant economic value [4]. Common carp is also produced and stocked into natural waters for angling purposes, and its colorful varieties (koi carp) represent one of the most expensive fish. A. salmonicida is recognized as a significant bacterial pathogen in ulcerative disease of common carp [5]. In cyprinid species, infection with this pathogen is known as "carp erythrodermatitis" because infection with this pathogen causes cutaneous hemorrhagic symptoms [6]. A. salmonicida-infected carp showed symptoms such as abnormal lethargy, swimming behavior, intra-abdominal fluid, multifocal necrosis, dropsy, infiltration of inflammatory cells in tissues, ulceration and hemorrhagic septicemia on fins and the ventral side of the body [2,7]. The mechanism of immune response to these bacteria in carp is not well understood.

Proteomics offers a comprehensive and large-scale analysis of proteins with direct interpretation of protein responses under the influence of disease, compared to genomic approaches, as proteins are the main effectors of most cellular processes. High-throughput comparative

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proteomic techniques such as 2D electrophoresis and liquid chromatography-mass spectrometry (LC-MS), including label-free techniques, isobaric tags (iTRAQ), tandem mass tag (TMT) have been successfully applied to study proteomic alterations during bacterial infection in various species of fish [8–13]. However, previous studies of the proteomic changes during bacterial infections in fish were performed mainly on liver, spleen, kidney, gill or intestinal mucosa, while reproductive tracts were not well studied. To the best of our knowledge, little is known about the regulation of the immune response in reproductive tissues at the protein level and the role of these organs in pathogen transmission and survival in fish.

Immunoregulation in the testis is characterized by a balance between immune suppression (or immune privilege) and the ability to react to infections. The regulation of immunity in the gonad in combination with the blood-testis barrier forms a special immunological environment that protects germ cells and responds to invading pathogens [14]. In fish, immune regulation in the gonad is poorly understood and restricted to few species such as seabream and rainbow trout [15]. As in mammals, the testes of gilthead seabream and European seabass express genes encoding anti- and proinflammatory cytokines, β-defensin, chemokines, antimicrobial peptides (AMPs), complement factors, immunoglobulins, and immune-related receptors [16]. Moreover, leukocytes such as granulocytes, macrophages, and lymphocytes were found in the testis and semen of various fish species [17,18]. Our previous studies demonstrated that *A. salmonicida* infection of carp males triggers a testicular immune response manifested by increased expression of proinflammatory *il1β* and inducible nitrogen oxide synthase (*inos*) [19]. However, the mechanisms by which carp might fight bacterial infections in the male reproductive system are not understood.

Carp seminal plasma is characterized by the dominance of immune-related proteins involved in the acute phase response (transferrin, alpha-1-antitrypsin, apolipoproteins, complement C3, Wap65) [20], the first rapid mechanism of protection against microbes and pathogens, repair of tissue damage and inactivation of proteases in fish [21]. Moreover, several other minor proteins participated in immune-related processes, indicating the essential function of seminal plasma in protecting spermatozoa against inflammation and infection. However, to our knowledge, the dynamics of these protein changes in response to bacterial infection have not yet been studied.

In the present study, we employed label-free proteomics to analyze the alterations in the protein profile of carp seminal plasma following *A. salmonicida* infection. The identification of differentially abundant proteins (DAPs) combined with the relevant bioinformatics analysis may contribute to further understanding the immune response mechanism within the testis following bacterial infection. Furthermore, some potential biomarkers were identified for assessing the infection of the carp reproductive tract.

2. Materials and methods

2.1. Infection experiment

The samples were collected during earlier described *A. salmonicida* infection of reproductively mature common carp males [19]. Briefly, six fish were intraperitoneally (i.p) injected with 200 µl of PBS containing 1 × 10^7 colony-forming units (CFU) of *A. salmonicida* per individual (AS group). The control (CON) fish (n = 6) received an i.p. injection of phosphate-buffered saline (PBS). Fish were kept for two days at 25 °C. Then, the carp were euthanized with 0.5 g L^{-1} MS222, and sperm and tissue from the testis, liver, kidney, and spleen were collected. The sperm was centrifuged at 2500 g for 15 min, and plasma supernatant was collected into a separate tube and stored at - 80 °C until further proteomic analysis. The tissues were placed in RNAlater (Sigma–Aldrich) and stored at - 80 °C until further mRNA expression analysis. Additional tissue fragments were collected in 4% buffered formalin and processed for histopathology by routine hematoxylin and eosin (H&E)

staining and evaluation of the pathological changes using a semi-quantitative scoring system. The experimental procedure was approved by the Local Ethical Commission in Krakow, Poland with allowance no. 49/2020.

2.2. Detection and quantification of bacterial DNA

Bacterial genomic DNA was isolated and quantified as described earlier [22]. Briefly, genomic DNA was isolated using QIAgen DNA Mini kit. The number of copies of Aeromonas DNA was quantified using Maxima SYBR Green mastermix and Aerom16S_qF1 GCGAAGGCGGCCCCCTGGACAAAGA Aerom16S_qR1 CCACGTCT-CAAGGACACAGCCTCCAAATC primers. The results are presented as genome copy numbers per 250 ng of isolated DNA.

2.3. Mass spectrometry

LC-MS analysis and sample preparation were performed at the Mass Spectrometry Laboratory at the Institute of Biochemistry and Biophysics PAS.

2.3.1. Sample preparation and measurement

Due to the low quality of the MS spectra obtained from the seminal plasma of one control and one infected fish, label-free quantitative proteomic analysis was performed on n = 5 for each group. Samples were diluted with ammonium bicarbonate buffer to a final concentration of 100 mM. Cysteine residues were reduced and blocked with 10 mM tris (2-carboxyethyl)phosphine through 1 h incubation at 60 °C followed by cysteine modification using 20 mM S-methyl methanethiosulfonate. Samples were digested overnight with 2 µg of trypsin/Lys-C mix (Promega) at 37 °C. Tryptic peptides were acidified with 0.1% formic acid (FA) and subsequently subjected to cleaning with 10 mg Oasis HLB columns (Waters). Samples were loaded and washed with 1 ml of 0.1% FA. Peptide elution from columns was executed using 200 µl of 0.1% FA and 80% acetonitrile (ACN). Aliquots were dried and resuspended in 50 µl 0.1% FA. Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific) was used to measure peptide concentration.

From each sample, 3 µg of peptide mixture was applied onto the LC-MS system composed of an ultraperformance liquid chromatograph (UPLC) (M-class, Waters) coupled to a Q Exactive mass spectrometer (Thermo Scientific) via a Flex nano electrospray ionization (ESI) ion source (Thermo Scientific). LC-MS analysis was carried out as described previously [23] with some modification such as ACN gradient (0–30% ACN in 160 min, 30–90% ACN in 3 min) for separation of peptides on C18 column, mass range of 300–1650 Da was used for MS scan and automated gain control (AGC) target value was 2e-5.

2.3.2. Data analysis

Data preprocessing and protein identification were performed with Mascot suite (Mascot Distiller 2.7 and Mascot Server 2.7, MatrixScience, London, UK). Files were searched against the database of *Cyprinus carpio* proteins (135,487 sequences; 77,807,391 residues) derived from the National Center for Biotechnology Information (NCBI) database (version 20210909). Offline mass recalibration was performed for each LC-MS run as described previously [24]. One missed cleavage was allowed, the enzyme was set to trypsin, the instrument was set to higher-energy collision dissociation (HCD), and the modifications allowed in the search were methylthio (C) (fixed) and oxidation (M) (variable). The confidence in peptide assignments was computed as q-values (threshold value 0.01) using a target/decoy strategy [25]. Peptides with q-values above the defined threshold, as well as single-peptide and subset proteins, were removed from further analysis. Recalibration and data filtering were performed with in-house Mscan software (http://proteom.ibb.waw.pl/mscan/).

2.3.3. Quantification

The common list of identified peptides was overlaid onto 2D heat-maps generated from LC-MS analyses using information on *m/z* value (measured and theoretical), elution time and match between theoretical and measured isotopic envelope shape [26]. For correctly matched peptides, the volume of the 2D fit of monoisotopic peaks was taken as an abundance estimate for further analysis. Scatter estimates were computed and data were explored in order to inspect variance levels prior to quantification (IQR and inspecting scatter plots are provided in Supplementary Fig. S1). Statistical analysis with DiffProt [24] software was performed on exported lists of peptide/protein identifications along with quantitative values. A total of 10^6 random peptide sets were generated with DiffProt for analysis, peptides were normalized with the LOWESS procedure, and peptide sets were clustered into single units when they were more than 90% identical. Quantitative values were further used for hierarchical clustering and unsupervised PCA.

2.4. Real time – quantitative polymerase chain reaction (RT-qPCR) analysis of gene expression

Total RNA was extracted from 20 mg of the liver, kidney, spleen, and testis collected from six control and six infected fish using TRI reagent (Sigma-Aldrich). The total RNA (200 ng) was treated with 1 U DNAse I for 30 min at 37 °C (Thermo Fisher Scientific) and transcribed to cDNA using random hexamer and oligo(dT)$_{18}$ primers and 100 U Maxima Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions.

RT-qPCR was performed in duplicate using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), specific primers, and cDNA (40 × diluted) according to the manufacturer's instructions. The sequences of the primers are listed in Supplementary Table S1. The changes in gene expression were analyzed using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method [27] as a reference for the geometric mean of two reference genes that encode 40S ribosomal protein S11 (*40S*) and elongation Factor 1 alpha (*ef1a*). The results were calculated and displayed as the fold change compared to the same tissue control.

2.5. Functional analysis

Ingenuity pathway analysis (IPA) software (Qiagen, CA, USA) was used to investigate the functional and canonical pathways enriched by the differentially abundant proteins. We used IPA Downstream Effector Analysis tool to predict the increase or decrease in downstream biological activities and functions that are likely to be affected by the differentially abundant proteins. Potential upstream regulators that may account for protein expression changes observed in our experimental dataset were predicted using the IPA Upstream Regulator Analysis tool based on Ingenuity Knowledge Base. Fisher's exact test and Benjamini-Hochberg multiple testing corrections were used to calculate statistical significance ($p < 0.05$). Gene Ontology (GO) annotations of differentially abundant proteins was carried out using the category 'biological function' and cellular component (ShinyGO v0.60). Protein–protein interaction network and protein–set enrichment analyses were performed using STRING 11.0 software (species *Homo sapiens*). The minimum interaction confidence score for the interaction sources was set to 0.4 for the networks.

2.6. Statistical analysis

For gene expression results, SigmaPlot 12.5 (Systat Software GmbH) was used for statistical analysis. The fold change results were tested with Normality Test (Shapiro-Wilk) and Equal Variance Test. Significant differences in the gene expression and pathological scores between *A. salmonicida*-infected fish and the control fish were assessed using a *t*-test at $p < 0.05$ in cases when the data were normally distributed or with the nonparametric Mann-Whitney Rank Sum Test at $p < 0.05$ when the

data were not normally distributed. The results are presented as box plots indicating the range of 25–75% in the box and maximum and minimum values by whiskers and showing all values as data points and a mean using GraphPad Prism 9.0 (GraphPad Software Inc.). The Z-score >2 is defined as the threshold of significant activation, whilst Z-score <-2 is defined as the threshold of significant increase.

3. Results

3.1. Bacteria load

No bacterial DNA was detected by qPCR in kidney, liver, spleen and testis of fish from the control group (Fig. 1). In *A. salmonicida* infected group the *A. salmonicida* DNA level increased to the highest level in spleen where mean of 563303 copies of bacteria DNA per 250 ng of isolated DNA were recorded. The lowest bacteria load was recorded in testis (16097 copies per 250 ng of isolated DNA). The kidney and liver bacteria loads were 201940 copies and 228468 copies per 250 ng of isolated DNA, respectively.

3.2. Expression of genes encoded pro-inflammatory cytokines

The expression of three immune-related genes, tumor necrosis factor alpha type 2 (*tnfa2*), interleukin 6a (*il6a*) and interferon gamma 2a (*ifng2a*), was studied using RT-qPCR. Bacterial infection led to higher expression of *tnfa2* in the head kidney (84-fold), liver (43009-fold), spleen (33-fold), and testis (43-fold) (Fig. 2A). The expression levels of *il6a* increased following *A. salmonicida* challenge in the liver (22-fold), spleen (21-fold), and testis (70-fold) compared to the control (Fig. 2B). The strong response of these immune markers in immune-related tissues is consistent with the systemic immune response to *A. salmonicida* infection in the animal used for other proteomic study [13]. The AS males showed lower expression of *ifng2a* the spleen (2-fold) than the control while its expression did not change in other tissue following infection (Fig. 2C).

3.3. Label-free quantitative proteomic analysis of the differences between seminal plasma proteins of A. salmonicida-infected and uninfected carp males

Using label-free quantitative proteomic profiling of carp seminal plasma from CON and AS males, 1402 proteins were identified with at least two unique peptides and a 1% FDR. Among the identified proteins, 44 were found to be differentially abundant (DAPs; *p* value < 0.05 and a ratio >1.5-fold difference). Twenty proteins were upregulated and 24 were downregulated following bacterial infection (Table 1, Supplementary Table S2). Hyaluronan and proteoglycan link protein 1-like isoform X2 (HAPLN1) and dipeptidase 1-like isoform X (DPEP1) were exclusively identified in seminal plasma of infected males, while ribosomal protein S6 kinase alpha-1 isoform X1 (RPS6KA1) and 40S ribosomal protein S23-like (RPS23) were unique for control males. As shown in Fig. 3A, hierarchical clustering analysis of the datasets displayed two clusters representing the AS and CON groups. Further principal component analysis revealed the clear separation of the CON and AS groups (Fig. 3B). The first principal component (PC1) distinguished 44.3% of the variance, and 25.1% additional variation was distinguished by the second principal component.

An additional search of mass spectra from seminal plasma collected from both groups against the *A. salmonicida* database allowed us to identify 21 *A. salmonicida* proteins in seminal plasma from infected males (Supplementary Table S3). These proteins were not identified in seminal plasma of control males.
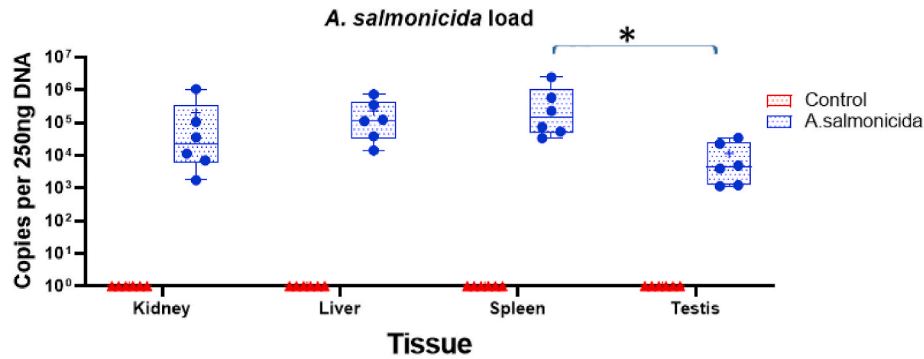
Fig. 1. *A. salmonicida* DNA in the kidney, liver, spleen and testis of carp measured by qPCR in control and infected males. Data are shown as mean genome copies per 250 ng of DNA (+SD) from n = 6 in control and *A. salmonicida* infected groups. *P < 0.05.
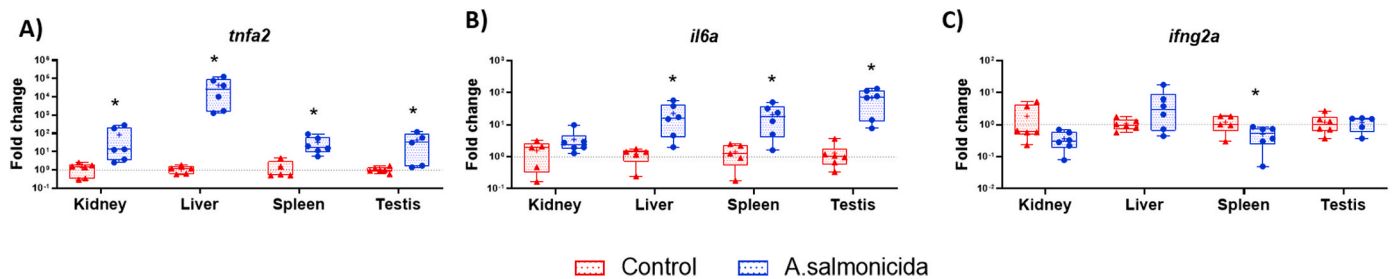

Fig. 2. RT-qPCR analysis of genes encoding pro-inflammatory cytokines in control and *A-salmonicida*-infected males. A-tumor necrosis factor alpha type 2 (*tnfa2*), B-interleukin 6 (*il6a*), *C*- interferon gamma 2a (*ifng2a*). Asterisk indicates significant difference between control and infected groups (P ≤ 0.05) in particular tissue.

3.4. Functional annotation of seminal plasma proteins changed following bacterial infection

3.4.1. IPA analysis

To further understand the functions of the identified proteins, we uploaded 41 (vitellogenin and saxitoxin- and tetrodotoxin-binding protein 2 were not found in the human database, complement C3a.3 and complement C3 had the same gene name) out of 44 differentially abundant proteins between CON and AS into IPA which revealed acute phase response signaling, LXR/RXR activation, creatine-phosphate biosynthesis, and germ cell-Sertoli cell junction signaling as the top canonical pathways, with several implicated molecules overlapping among the different pathways (Table 2). The top molecular and cellular function categories included lipid metabolism, molecular transport, small molecule biochemistry, protein synthesis and vitamin and mineral metabolism (Table 2). The top disease and disorder categories included Inflammatory Response, Metabolic Disease, Organismal Injury and Abnormalities and Hematological Disease. Fig. 4 presents the specific functions/disease with their predicted activation state (activated -orange or inhibited – blue; darker colors indicate higher z-scores) and associated molecules within the top-ranked categories: Lipid metabolism, Inflammatory response, Molecular transport and Hematological system development (Table 2). Interestingly, IPA Downstream Effects analysis identified one canonical pathway and two functions presented z-scores: activation of acute phase response signaling (z score 2.249), activation of hemostasis (z score 2.215) and activation of secretion of molecules (z score 2.032) following infection. The top enriched networks were associated with: (i) cell cycle, cell morphology, cellular assembly and organization (score 48), consisting of 21 DAPs; (ii) lipid metabolism, small molecule biochemistry, and molecular transport (score 24), consisting of 12 DAPs; and (iii) cell morphology, cell-to-cell signaling and interaction (score 14), consisting of 6 proteins (Fig. 5). Complete sets of all the IPA results are presented in Supplementary Table S4.

We also searched in silico for upstream regulators of differentially abundant proteins. In the AS males, IL6 (z score: 2.33; $p = 1.11E-05$) and platelet-derived growth factor (PDGF, z score 2.00; p -value 3.15E-0.3) were identified as potential activated upstream regulators based on the changes in abundance of several proteins involved in immune response. IL6 leads to activation of ApoA1, A2M, C3, CP, FGA, FN1, LCAT and inhibition of APOE and DPP3 while activation of PDGF activates C3, CP, FN1 and inhibits YWHAH (Supplementary Fig. S2). The expected regulation effect was consistent with changed in protein abundance observed in our proteomic study with exception of LCAT which decreased following infection. The mechanistic network generated by IPA for IL6 showed that IL6 activated TNF alpha, NFKBIA and the NF-kB complex and affected five other intermediate regulatory molecules that were connected to 17 DAPs (Supplementary Fig. S3).

3.4.2. GO enrichment and protein-protein interaction analysis

GO Biological process analysis revealed enrichment of triglyceride-rich lipoprotein particle remodeling, positive regulation of cholesterol esterification, immune system processes, regulated exocytosis, and cell substrate adhesion. According to the GO Cellular complement analysis, most of the proteins were localized to extracellular vesicles and space, the secretory granule lumen, high-density lipoprotein particles, the extracellular matrix (ECM) and the DNA packing complex (Supplementary Figs. S4A and B, Supplementary Tables S5 and S6). Very high confidence interactions (interaction score >0.8) were observed between proteins involved in triglyceride-rich lipoprotein particle remodeling (LCAT, CEPT, APOA2, APOA1, APOE), defense response (A2M, C3, FGA, FN1, APOA2, APOA1, APOE) and between histones using STRING analysis (Supplementary Fig. S4C). The analysis of enriched biological processes provided by STRING revealed the association of differentially abundant proteins in triglyceride-rich lipoprotein particle remodeling (p value 4.01E-06, pink nodes), posttranslational protein modifications (p value 8.6E-05, yellow nodes) and defense response (p value 9.1E-05, blue nodes) (Supplementary Fig. S4C).

The functional analysis of identified in this study proteins with the use of bioinformatics software such as IPA, String and ShinyGo based on

Table 1

List of differentially abundant proteins between *A. salmonicida*-infected (AS) and control (CON) males of carp.

Protein name	Gene symbol	Human homolog	Accession number	P-value	Fold change AS/CON
Hyaluronan and proteoglycan link protein 1-like isoform X2	*hapln1*	HAPLN1	KTG38360.1	NA	only in AS
Dipeptidase 1-like isoform X	*dpep1*	DPEP1	KTF96654.1	NA	only in AS
Parvalbumin alpha	*pvalba*	PVALB	XP018922924.1	0.00112	15.01
Creatine kinase M-type	*ckmb*	CKM	KTF81044.1	0.00020	39.09
Eosinophil peroxidase-like	*epx*	EPX	XP018925078.1	0.00846	35.54
PREDICTED: ependymin-like isoform X1	*epdr1*	EPDR1	XP018950094.1	0.00568	12.84
Vitellogenin	*vtg*		AAL07472.1	0.00814	11.06
Complement C3-like (similar to c3b)	*c3b*	C3	KTF92880.1	0.02387	10.52
Complement C3a.3, fragment	*c3a3*	C3	KTG40750.1	0.04093	8.56
Alpha-2-macroglobulin family, partial	*a2m3*	A2M	KTF87003.1	0.00829	7.72
Alpha-*N*-acetylgalactosaminidase precursor	*naga*	NAGA	KTG04161.1	0.02378	4.47
Saxitoxin and tetrodotoxin-binding protein 2-like	*psbp2*		XP018961212.1	0.04420	4.08
Complement component C7-like	*c7a*	C7	XP018919401.1	0.00163	3.83
Apolipoprotein A-I	*apoa1*	APOA1	AHJ79064.1	0.00917	2.64
Apolipoprotein A-II precursor	*apo14*	APOA2	XP018931187.1	0.00112	2.56
Inter-alpha-trypsin inhibitor heavy chain H2	*itih2*	ITIH2	KTF86194.1	0.03223	2.35
Cholesteryl ester transfer protein	*cetp*	CETP	KTG31753.1	0.00874	2.27
Fibronectin isoform X1	*fn*	FN1	KTF75090.1	0.00957	2.04
Fibrinogen alpha chain precursor	*fga*	FGA	KTF87998.1	0.02173	2.03
Ceruloplasmin	*cp*	CP	KTF79664.1	0.00976	1.95
Creatine kinase, brain b	*ckbb*	CKB	KTG39582.1	0.03164	−2.65
Apolipoprotein Eb	*apoeb*	APOE	KTG31224.1	0.03275	−2.71
Actinin, alpha 4	*actn4*	ACTN4	KTG32258.1	0.00311	−3.07
Transketolase	*tktb*	TKT	KTG42770.1	0.00020	−3.26
Glutaredoxin 3-like	*glrx3*	GLRX3	XP018934300.1	0.00261	−4.23
coiled-coil domain-containing protein 62-like isoform X5	*ccdc62*	CCDC62	XP018931733.1	0.008423	−4.27
Tubulin beta-4B chain isoform X3	*tubb4b*	TUBB4B	XP018934601.1	0.04378	−5.37
Histone H4-like	*h4*	H3C1	XP018963044.1	0.00020	−5.73
14-3-3 protein beta/alpha-A-like	*ywhab*	YWHAH	XP018979902.1	0.00020	−5.79
Histone H2A.Z.7	*h2az2b*	H2AZ2	AFV36374.1	0.00035	−6.05
Actin, cytoplasmic 2	*actb1*	ACTG1	KTF76943.1	0.00020	−7.03
Histone H2B-like	*hist2h2l*	H2bc1	XP018977972.1	0.00020	−7.11
Elongation factor 1-alpha 1-like	*eef1a1b*	EEF1A1	XP018950712.1	0.00127	−8.37
Dipeptidyl peptidase 3-like	*dpp3*	DPP3	XP018929389.1	0.001365	−8.42
Cytochrome *b-c*1 complex subunit 2, mitochondrial, partial	*uqcrc2b*	UQCRC2	KTF91707.1	0.000753	−8.45
C-factor-like	*zgc:92161*	LCAT	KTG42627.1	0.01670	−10.4
Adenylyl cyclase-associated protein 1-like	*cap1*	CAP1	XP018940001.1	0.00020	−10.84
Mimecan	*ogn*	OGN	KTG33060.1	0.00947	−11.65
Ictacalcin-like	*icn*	S100A4	XP018974737.1	0.00020	−13.48
Histone H1-like	*zgc:163061*	H1-5	KTF71206.1	0.01917	−17.1
Histone H2A-like	*h2afx1*	H2AC11	XP018967130.1	0.00942	−17.92
COP9 signalosome complex subunit 2	*cops2*	COPS2	KTF83911.1	0.00028	−50.76
Ribosomal protein S6 kinase alpha-1 isoform X1	*rps6ka1*	RPS6KA1	KTG06164.1	NA	Only in CON
40S ribosomal protein S23-like	*rps23*	RPS23	KTG01543.1	NA	Only in CON
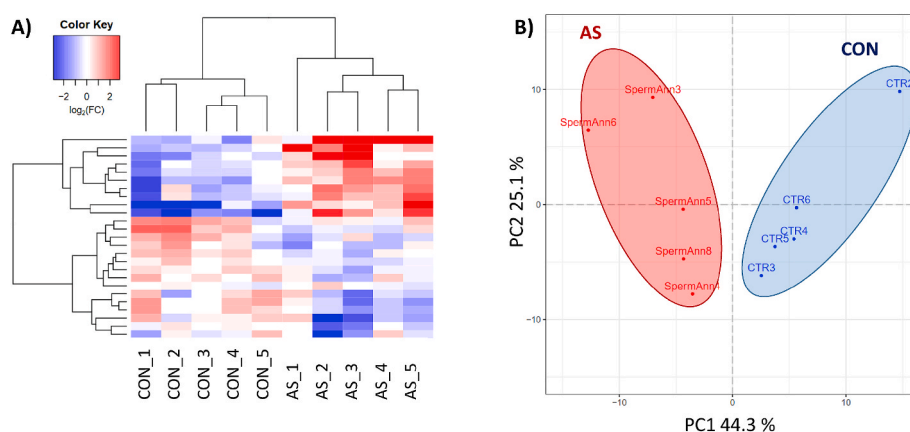
Fig. 3. Heatmap (A) and principal component analysis (B) of differentially abundant seminal plasma proteins between control (CON) and *A. salmonicida*-infected (AS) carp males.

human database should be treated with caution.

3.5. RT-qPCR analysis of differentially abundant proteins

The expression of 20 genes encoding the differentially abundant

proteins during *A. salmonicida* infection were studied using the RT-qPCR technique in the liver, head kidney, spleen, and testis (Fig. 6). The full name of the genes are indicated in Table 1 and Supplementary Table S1. In the kidney four genes showed higher relative expression levels following infection: complement C3b (*c3b*; 11-fold), alpha-2-

Table 2

Ingenuity pathway analysis of differentially abundant seminal plasma proteins between uninfected and *A. salmonicida*-infected carp males.

Categories	P value	Proteins
Top Canonical Pathways		
Acute Phase Response Signaling	8.78E-10	A2M,APOA1,APOA2,C3,CP,FGA, FN1,ITIH2
LXR/RXR Activation	1.61E-09	APOA1,APOA2,APOE,C3,CETP,FGA, LCAT
Complement and coagulation system	1.73E-09	A2M,C3,FGA,C7,FN1
Creatine-phosphate Biosynthesis	1.91E-09	CKB,CKM
Germ Cell-Sertoli Cell Junction Signaling	2.14E-04	A2M,ACTG1,ACTN4,TUBB4B
Molecular and cellular functions		
Lipid Metabolism	2.34E-11-1.73E-03	A2M,APOA1,APOA2,APOE,C3, CETP,CP,DPEP1,EEF1A1,FN1,LCAT, NAGA,PVALB, YWHAH
Molecular Transport	2.34E-11-1.73E-03	A2M,ACTN4,APOA1,APOA2,APOE, C3,CAP1,CETP,CKM,CP,DPEP1, EEF1A1,FGA,FN1,HOOK3,LCAT, PVALB,S100A4,YWHAH
Small Molecule Biochemistry	2.34E-11-1.73E-03	A2M,APOA1,APOA2,APOE,C3, CAP1,CETP,CKB,CKM,CP,DPEP1, EEF1A1,FN1,LCAT, NAGA,PVALB, YWHAH
Protein Synthesis	1.38E-10-9.74E-04	APOA1,APOA2,APOE,C3,CETP,CP, DPP3,EEF1A1,FGA,FN1,H1-5,H3C1, ITIH2,LCAT,RPS23,S100A4
Vitamin and Mineral Metabolism	1.09E-07-1.73E-03	A2M,APOA1,APOA2,APOE,C3, CETP,CKM,FN1,LCAT, PVALB, YWHAH
Physiological system development and functions		
Hematological System Development and Function	5.86E-06-1.73E-03	A2M,ACTN4,APOA1,APOA2,APOE, C3,CP,DPEP1,EPX,FGA,FN1,H3C1, LCAT,S100A4,UQCRC2
Lymphoid Tissue Structure and Development	1.74E-05-1.73E-03	APOE,C3,FN1
Organ Morphology	1.74E-05-1.73E-03	A2M,ACTN4,APOA1,APOA2,APOE, C3,CETP,CKB,CKM,CP,DPP3,FGA, FN1,GLRX3,LCAT,OGN,PVALB, S100A4,TKT
Renal and Urological System Development and Function	1.75E-05-1.73E-03	ACTN4,APOA1,APOE,C3,FGA,FN1, LCAT,TKT
Immune Cell Trafficking	3.46E-05-1.64E-03	A2M,ACTN4,APOA1,APOE,C3, DPEP1,EPX,FGA,FN1,LCAT,S100A4
Disease		
Inflammatory Response	1.02E-07-1.73E-03	A2M,ACTN4,APOA1,APOA2,APOE, C3,C7,CAP1,CKB,CKM,CP,DPEP1, EEF1A1,EPX,FGA,FN1,H2BC1, LCAT,RPS6KA1,S100A4,TKT, TUBB4B
Hematological Disease	1.14E-07-1.73E-03	APOA1,APOA2,APOE,C3,CETP, CKM,CP,FN1,LCAT,S100A4,TUBB4B
Metabolic Disease	1.14E-07-1.73E-03	A2M,APOA1,APOA2,APOE,C3,C7, CETP,CKM,COPS2,CP,DPEP1,EPX, FGA,FN1,HAPLN1,LCAT, NAGA, OGN,S100A4,TKT,UQCRC2
Organismal Injury and Abnormalities	1.14E-07-1.73E-03	A2M,ACTG1,ACTN4,APOA1, APOA2,APOE,C3,C7,CETP,CKB, CKM,COPS2,CP,DPEP1,DPP3, EEF1A1,EPDR1,EPX,FGA,FN1, GLRX3,H3C1,HAPLN1,HOOK3, LCAT, NAGA,OGN,PVALB,RPS23, RPS6KA1,S100A4,TKT,TUBB4B, UQCRC2,YWHAH

macroglobulin 3 (*a2m3*; 6-fold), complement C7a (*c7a*; 30-fold) and cholesteryl ester transfer protein (*cetp*; 11-fold), while hyaluronan and proteoglycan link protein 1-like (*hpln1*), dipeptidase 1-like (*dpep1*), and vitellogenin (*vtg*) expression was downregulated (by 4-fold, 86-fold and 10-fold, respectively) in AS-infected males. In the liver, bacterial infection led to higher expression of 11 genes: *dpep1* (405-fold), eosinophil peroxidase-like (*epx*, 655-fold), ependymin-like 1 (*epdr1*; 19-fold), *c3b* (10-fold), alpha-*N*-acetylgalactosaminidase-like (*naga*; 32-fold),

saxitoxin and tetrodotoxin-binding protein 2-like (*psbp2*; 57-fold), *c7a* (30-fold), *cetp* (30-fold), fibronectin (*fn*; 5-fold), fibrinogen alpha chain (*fga*; 24-fold), and ceruloplasmin (*cp*; 82-fold), while in the spleen, the expression levels of 7 genes were upregulated following infection: *exp* (10-fold), *c3b* (24-fold), *c7a* (449-fold), *cetp* (4-fold), *fn* (4-fold), *fga* (17-fold), and *cp* (4-fold). In the testis, the expression levels of four genes were upregulated: creatine kinase, M type (*ckmb*; 7-fold), *c3b* (4-fold), *c7a* (523-fold), and *fn* (3-fold), while the expression of 5 genes was downregulated: *hapln1* (4-fold), complement C3a3 (*c3a3*; 2-fold), *a2m3* (3-fold), apolipoprotein A-I (*apoa1*; 6-fold), and apolipoprotein 14 kDa (*apo14*; 3-fold) following bacterial infection compared to the control. We did not observe differences in the expression levels of parvalbumin alpha (*pvalba*) and inter-alpha-trypsin inhibitor heavy chain H2 (*itih2*) between infected and control males. The same direction of changes between the protein and mRNA levels was found for CKM, C7, C3, FN1, while the opposite direction (upregulation at the protein level and downregulation at the mRNA level) was demonstrated for A2M3, APOA1, APOA2, HAPLN1 and C3, partial.

Moreover, the expression levels of two genes predicted by IPA upstream regulators nuclear factor NF-kappa-B p105 subunit-like (*nfkb p105*) and platelet-derived growth factor beta polypeptide b (*pdgfbb*) were analyzed. The AS males showed higher expression of *nfkb p1*05 by 6-fold in the liver and lower expression of *pdgfb* in kidney (3-fold) than the control (Supplementary Fig. S5).

*3.6. Histopathological tissue studies*

A semiquantitative evaluation of histopathological changes induced by *A. salmonicida* infection is presented in Fig. 7 and Supplementary Table S7. In the kidney of infected fish, the tubules showed various degrees of degradation, and the parenchyma between tubules was strongly hyperemic in some spots. There are moderate amounts of melanomacrophages and reaction centers in the kidney of the infected fish. Some areas showed accumulations of inflammatory cell infiltrates. While the liver of fish in the control group was largely unchanged, the parenchyma in the infected fish was severely hyperemic. In the spleen of the control fish, high levels of melanomacrophages and the formation of reaction centers were observed. The splenic parenchyma of the infected fish showed a very high number of melanomacrophages and a widespread formation of reaction centers. The testicular tissue of the infected fish was heavily pervaded by hyperemic areas in the parenchyma. The observed pathological changes in the tissue morphology (Supplementary Table S7) confirm the successful infection challenge.

4. Discussion

Based on our previous results, which demonstrated that *A. salmonicida* infection triggers testicular immunity manifested by an increase in *inos*, *il1β* and *wap65-2* expression and crosses the blood-testis barrier [19], we assumed that the changes in carp seminal plasma proteins were related to infection. Our proteomic results confirmed this hypothesis and led to the identification of 44 seminal plasma proteins that showed significant differential abundance following bacterial infection. To the best of our knowledge, this is the first report indicating changes in a wide range of seminal plasma proteins following bacterial infection in fish.

4.1. Expression of pro-inflammatory cytokines in tissues

In our study we selected three immune-related genes (*il6a, tnfa2, ifng2a*) which are markers important for characterization of the immune response to infection in fish. IL6 despite being pro-inflammatory cytokine together with IFN, has also anti-inflammatory properties. TNF using different mechanisms of action than interferons and interleukins promotes phagocytosis and nitric oxide production in teleost during bacterial infection. In our study expression of *il6* and *tnfa2* were
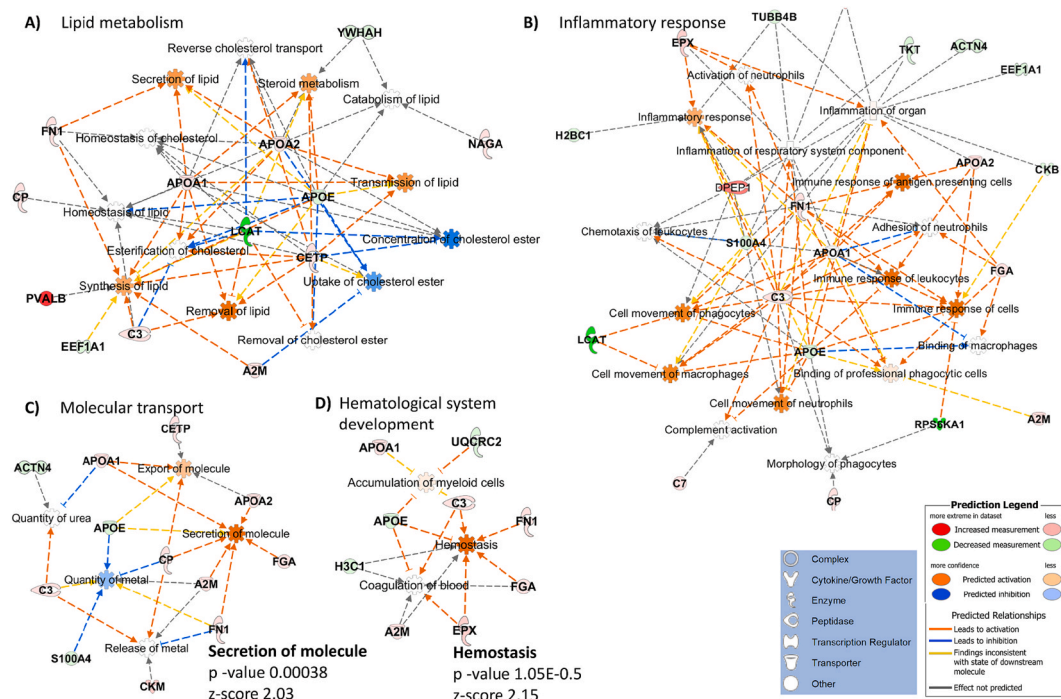
Fig. 4. Top-ranked specific functional IPA categories: lipid metabolism (A), inflammatory response (B), molecular transport (C) and hematological system development (D). Nodes in red are up-regulated in infected group, and green color shows down-regulation. Nodes shapes represent the functional class of the proteins. The edges connecting the proteins to the respective functions represent the predicted/indirect relationships (broken edges). An orange line indicates predicted upregulation, whereas a blue line indicates predicted downregulation. A yellow line indicates expression being contradictory to the prediction. Gray line indicates that direction of change is not predicted. Edges are marked with symbols to represent the relationship between protein and function (arrow = activation, suppression dash = inhibition). The functions are colored by their predicted activation state: activated (orange) or inhibited (blue). Darker colors indicate higher absolute Z-scores. The legend explaining node shape and edge type is given in the bottom right panel.

significantly upregulated in immune relevant tissue (spleen, head kidney and liver) and testis following *A. salmonicida* infection which agrees with previous data showing strong upregulation of *il6* and *tnfa2* in spleen and head kidney of crucian carp following *A. salmonicida* treatment [28]. On the other hand, we observed down-regulation of *ifng2a* expression in spleen following infection while its expression did not change in other tissue. Our results are consistent with previous studies indicating decreased expression of *ifng2a* in fugu spleen while its expression did not change in pufferfish kidney in response to bacterial infection [29]. Extensive studies demonstrated anti-viral properties of IFNa2 in different fish species but it remains enigmatic against bacterial pathogens in fish [30]. We would like to underline that IL6 and TNFA2 were not detected at protein level by LC-MS analysis of seminal plasma probably due to low concentration of these pro-inflammatory cytokines in seminal plasma.

4.2. Upregulation of acute phase proteins

Most seminal proteins altered upon infection were related to innate immune defense, such as acute phase response signaling and the complement and coagulation system. Moreover, in the testis, systemic *A. salmonicida* infection resulted in the upregulation of proinflammatory cytokines such as IL6 and TNFα, both associated with the induction of acute phase proteins (APPs), including A2M, CP, C3, FGA, and ITIH3. These proteins are recognized as positive APPs whose concentration increases significantly during inflammation. The upregulation, both at the protein and mRNA levels, complement components C3 and C7 indicates activation of the complement system in carp seminal plasma after *A. salmonicida* infection. Upon infection, enrichment of APOA1 and APOA2 coincides with their action as positive APPs in many teleost species (carp, channel catfish, Atlantic halibut) with direct antimicrobial properties [31,32], likely through the mechanism of LPS binding or

together with FGA (which was proposed as a biomarker of *A. salmonicida* infection in Atlantic salmon [11]) as an immune regulator involved in complement regulation [33]. The increased level of APPs in carp seminal plasma is consistent with their upregulation in transcriptomic and proteomic studies of other tissues during bacterial infections in different fish species [11,13,16,23,34–36]. Our results suggest the involvement of APPs in neutralizing *A. salmonicida*, minimizing the extent of tissue damage, and participating in tissue regeneration within the testis and the possible utility of these proteins as diagnostic tools in carp reproductive tract infections.

4.3. Proteins involved in lipid metabolism

Bacterial infection also led to alterations in proteins associated with lipid metabolism, including apolipoproteins (APOA1, APOA2, the major protein constituents (90%) of HDL and APOE) and enzymes involved in high-density lipoprotein (HDL) particle remodeling, such as down-regulated lecithin cholesterol acetyltransferase (LCAT) and upregulated cholesteryl ester transfer protein (CETP). LCAT converts free cholesterol into cholesteryl ester that migrate to the core of the HDL, while CETP facilitate transfer of cholesterol esters from HDL to LDL or VLDL [37]. The upregulation of CETP may result in elevation of triglyceride levels in semen and blood, as was indicated for mammals during infection and inflammation [38] and in our present study. HDL, in addition to its role in lipid metabolism, plays an important role as an antioxidant and influences leukocyte reactivity during infection and inflammation and together with its apolipoproteins displayed antibacterial activity [31,39, 40]. Moreover, during infection in mammals, HDL particles are selectively remodeled by LCAT to maximize the net HDL particle surface area (phospholipid content) for LPS binding. The present study also suggests that LXR/RXR signaling pathways can be one of the potential molecular mechanisms underlying the changes in proteins involved in lipid
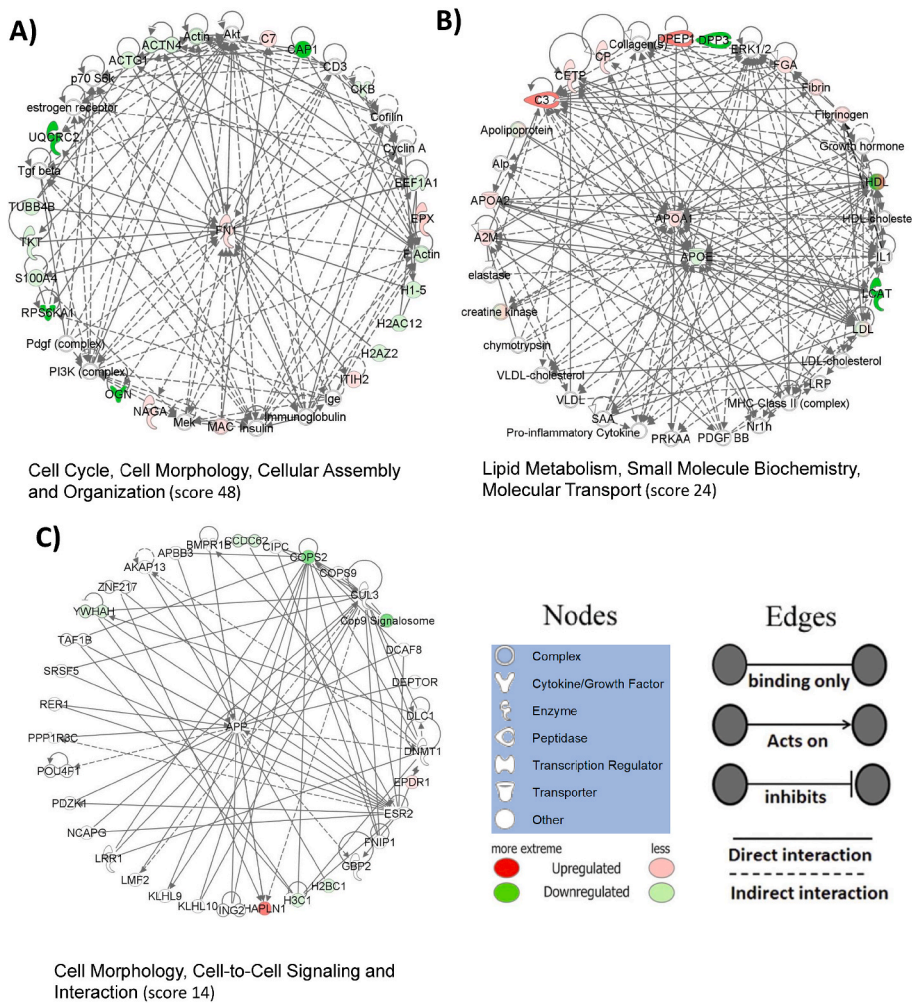
Fig. 5. The top scoring IPA networks A) Cell Cycle, Cell Morphology, Cellular Assembly and Organization, B) Lipid Metabolism, Small Molecule Biochemistry, Molecular Transport, C) Cell morphology, Cell-to-cell signaling and interaction associated with differentially abundant seminal plasma proteins. The IPA generates networks of differentially expressed proteins and their potential association with other known proteins. Nodes represent proteins and lines show the relationship (solid and dotted lines represent direct and indirect association, respectively). Nodes shapes indicate protein's primary function. The intensity of the node color indicates the degree of the up-regulation (red) or down-regulation (green) of significant proteins following infection with A. salmonicida. Non-color nodes are added by the IPA through relationships with other molecules. Edges are marked with symbols to represent the relationship between nodes (arrow = activation, suppression dash = inhibition, line only = binding). The legend explaining node shape and edge type is given in the bottom right panel.

metabolism since this pathway controls apolipoproteins and CETP gene expression. Our results indicate that lipoprotein remodeling and lipid changes might play a role in the reproductive tract in carp during bacterial infection.

4.4. Adhesion proteins

The bioinformatic analysis of DAPs clearly supports their role in the cell-extracellular matrix and cell-cell adhesion, playing a crucial role in initiating and supporting an effective immune response against pathogens. Interestingly, DPEP1, a zinc-dependent metalloproteinase, was exclusively identified in *A. salmonicida*-infected males. DPEP1, in addition to its dipeptidase activity, was recently recognized in mammals as a major adhesion receptor for neutrophil sequestration in the lung and liver [41]. These data suggest the involvement of the DPEP1-mediated adhesion mechanism by which neutrophils can be sequestered into the testis during infection in carp. Bacterial infection also led to enrichment of fibronectin 1 (FN1), an extracellular matrix glycoprotein that plays vital roles in cellular adhesion modulation between leucocytes: monocytes, eosinophils and the extracellular matrix [42]. In addition, we observed alterations in actin and actin binding proteins, such as actinin and adenosyl cyclase-associated protein 1 involved in actin-based cell to cell and cell to the ECM adhesions during infection and inflammation and in the regulation of the assembly and remodeling of epithelial junctions and the establishment of tissue barriers [43]. Moreover, ECM is a preferential target for bacterial adhesion, and targeting ECM proteins for adherence is, therefore, one of the major strategies for pathogen

colonization and host invasion [44]. Our results confirmed dramatic changes in ECM proteins, such as upregulation of HAPLN1 and down-regulation of mimecan, also known as osteoglycin (OGN). HAPLN1 regulates inflammation and tissue repair through the release of inflammatory cytokines and regulates inflammatory cell recruitment and cell migration [45,46], while OGN is a small leucine-rich proteoglycan involved in many biological processes, including cellular growth, angiogenesis, pathogen binding and inflammation. The changes in HPLN1 and OGN upon bacterial infection coincide with significant modifications of ECM composition and turnover, which have a profound impact on the specific signals that the ECM conveys to immune cells at the forefront of infection and inflammation. Altogether, our findings suggest that DPEP1- and FN1-mediated adhesion mechanisms and ECM remodeling are essential strategies of carp responses to pathogens. These changes may alter the epithelial barrier integrity, affecting ECM functions in regulating spermatogenesis and blood-testis barrier integrity.

Furthermore, we observed upregulation of ependymin, a fish-specific protein recognized as the predominant constituent of brain extracellular fluid in teleost fish involved in neuronal growth and regeneration, maintaining calcium homeostasis in the brain and cold adaptation [47]. The role of ependymin-related proteins outside the central nervous in mammals is proposed to be cell-cell/cell-matrix adhesion in a calcium-dependent manner, contractility, and regeneration [48], but its specific function is still unclear. Our results suggest that fish ependymin might be involved in the immune response against bacterial infection in testis; however, its function in this mechanism will be an interesting avenue of future research.
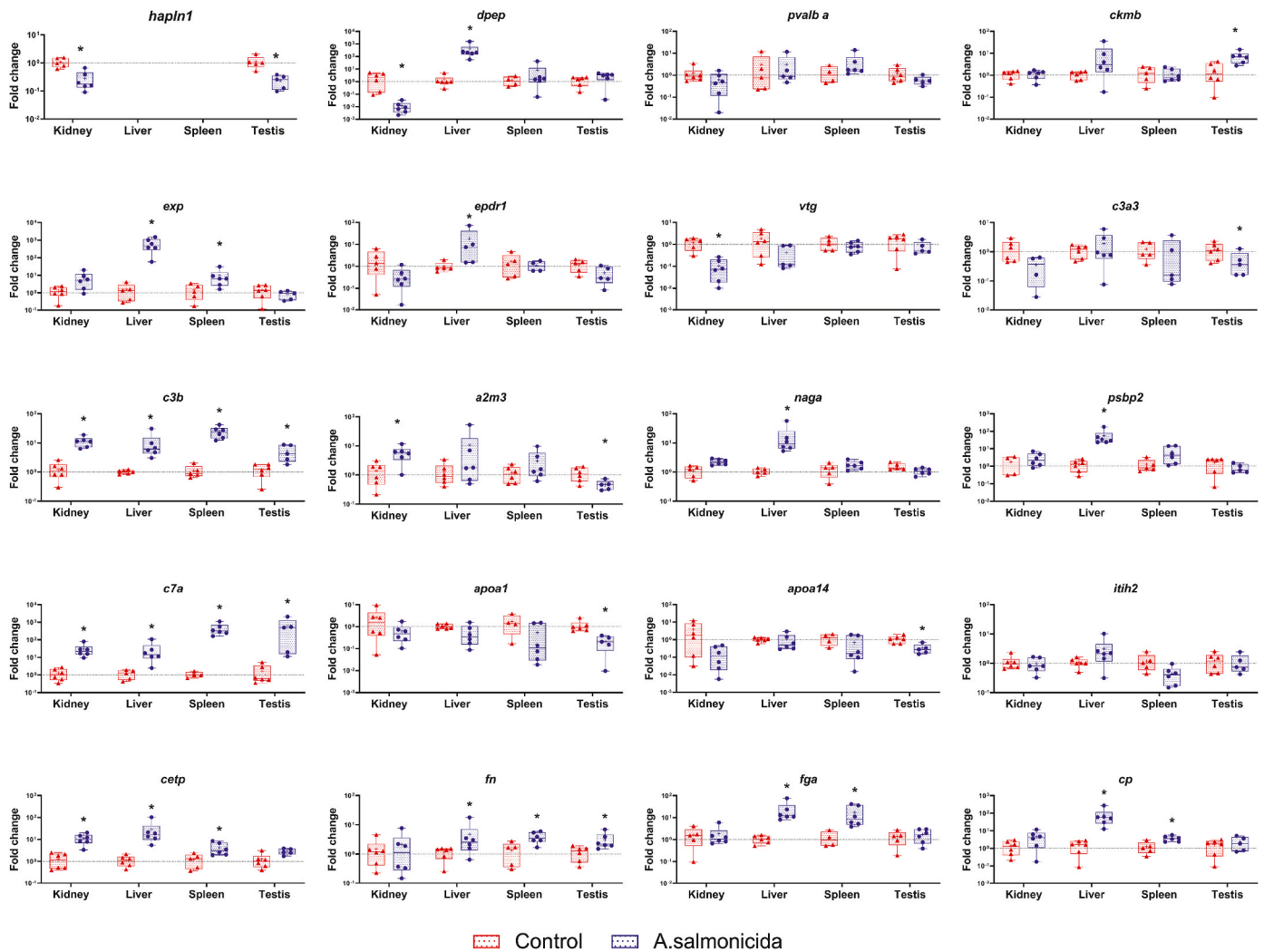
Fig. 6. RT-qPCR analysis of genes encoding the differentially expressed proteins with higher abundance following *A. salmonicida* infection. Detailed information on protein names and abbreviations can be found in Table 1. Asterisk indicates significant difference between control and infected groups (P ≤ 0.05) in particular tissue.
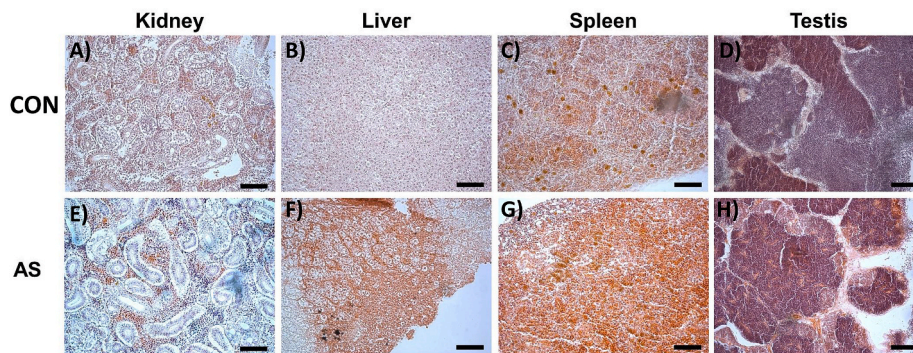

Fig. 7. Photomicrographs showing sections of the kidney, liver, spleen and testis of common carp. A and D control (CON; PBS-treated males), E to H fish challenged with *A. salmonicida* (AS). Staining: hematoxylin and eosin.

4.5. Immune-related proteins

Interestingly, our results indicated the dramatic enrichment of alpha-*N*-acetylgalactosaminidase (NAGA) following bacterial infection. NAGA together with CRP is a marker for inflammation in human that is often elevated in viral infections, cancer, and other chronic conditions [49, 50]. Its elevation was associated with immune system deficiency since

NAGA prevents the formation of the regulatory protein Gc protein-derived macrophage activating factor (GcMAF), an immune stimulant cytokine [49,50]. The immune role of NAGA, to our knowledge, has not been reported in fish, and our data suggest the possible utilization of this protein as a biomarker of inflammation/disease in the carp reproductive system.

Our results indicate the involvement of eosinophil peroxidase (EPX),

a major eosinophil-specific granular protein, in the immune response against *A. salmonicida* in carp testes. EPX is considered a molecular marker of eosinophil activity and is also known as an inflammatory marker. EPX displays numerous immunological activities, such as bactericidal activity and innate factors that protect the host from bacterial endotoxins [51]. EPX activity in teleosts have been reported in response against parasites, viruses and bacteria [13,52–55]. The marked increase in EPX in carp seminal plasma suggests eosinophil degranulation in response to bacteria and indicates the participation of eosinophils in immune defense in the testis.

Bacterial infection also led to an increase in fish-specific protein, saxitoxin- and tetradotoxin-binding protein 2, which are capable of binding to the neurotoxins saxitoxin and tetrodotoxin produced by bacteria. This protein constitutes a toxin-tolerant system in pufferfish [56]. It was also recently identified in pikeperch seminal plasma [23]. The enrichment of saxitoxin- and tetrodotoxin-binding protein 2 in carp seminal plasma following bacterial infection suggests its role in detoxification mechanisms in protecting spermatozoa and reproductive tract tissue against tetrodotoxin produced by *A. salmonicida* [57].

4.6. Energy metabolism-related proteins

Bacterial infection affected energy metabolic pathways such as the creatine/phosphocreatine pathway (CK-M, CK-B), oxidative phosphorylation (mitochondrial cytochrome *bc*1 complex subunit 2 (UQCRC2) and pentose phosphate pathway (TKT) which coincide with the dramatic shift in innate cellular metabolism occurring during the early immune response [58]. Such shifts in energy supply and demand result from a combination of profound recruitment of inflammatory cells (monocytes/macrophages, neutrophils) and high proliferation rates of lymphocyte [59]. We observed the opposite direction in the changes of two cytosolic creatine kinase (CK) isozymes: CK-M increased (39-fold), while CK-B decreased (2.65-fold) in abundance following infection. CK is poised to function as a conduit for rapid adenosine triphosphate (ATP) generation for epithelial junction assembly and barrier restitution during recruitment of immune cells to sites of inflammation [60]. CK has also been identified as an acute phase protein and bacteriostatic factor with lectin-like activity [61]. Our results strongly suggest that CK-M is a major isoform involved in these functions during bacterial infection. Moreover, we observed a decrease in transketolase (TKT), an enzyme in the nonoxidative branch of the pentose phosphate pathway (PPP) that serves as a bridge linking the PPP and glycolysis. TKT deficiency causes a significant reduction in the conversion from glucose to pyruvate, leading to insufficient carbohydrate-derived energy supply [62], which suggests a disruption in the interconnection between metabolic pathways during infection. We also found a decrease in UQCRC2 (complex III), a central component of the respiratory electron transport chain, which indicates dysregulation of mitochondrial metabolism following bacterial infection. These changes can reflect the shift in energy production from OXPHOS, a major energy source in quiescent cells of the innate immune system, to glucose metabolic pathways upon immune cell activation [63].

4.7. Calcium-binding proteins

The calcium concentration in extracellular fluid increases markedly at sites of infection and inflammation [64], which corroborates our finding indicating changes in Ca^{2+}-binding proteins, such as upregulation of parvalbumin (PV) (52-fold) and downregulation of ictacalcin (13-fold), following bacterial challenge. Ictacalin is a fish-specific member of the S100 family absent in mammals and has not been identified in fish semen yet, whereas parvalbumin was detected in high amounts in both carp spermatozoa and seminal plasma [65,66]. Both proteins are members of the EF-hand superfamily but differ in calcium binding mechanism. Parvalbumin functions as a Ca^{2+} buffer or transporter, while ictacalcin mediates Ca^{2+} intracellular signaling [67]. The

downregulation of ictacalcin corroborates previous studies indicating the reduction of this protein in the spleen and head-kidney of *Y. ruckei*-infected rainbow trout and in the skin mucus of *E. ictaluri*-infected yellow catfish [35,51]. To date, the role of carp semen PV has been indicated in the Ca^{2+}-mediated mechanism of sperm activation in carp [65] and upregulation of PVs was also observed in the gills of *A. hydrophila*-infected zebrafish [8]. As changes in the mRNA expression of gene encoded PV was not observed, we assumed that the dramatic increase in PV levels in seminal plasma may result from spermatozoa leakage, as it contains high amounts of PV [65]. Overall, our results suggest that bacterial infection led to calcium homeostasis alterations in carp semen, including both calcium buffering and calcium signaling systems.

4.8. Upregulation of vitellogenin

A notable study finding was the strong upregulation of vitellogenins (vtg1-3) in carp seminal plasma following *A. salmonicida* challenge. These proteins were also detected at lower levels in four control males (Supplementary Table S2). 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, *vtg* gene is silent; however, it is also produced by fish males exposed to exogenous estrogen [68] and can be related to testis development [23]. Vtg in fish, in addition to its nutritional role, is also involved in defense responses with bacterial-binding and inhibiting activities [69–71]. Later suggested that Vtg is a novel candidate immune-relevant molecule involved in the acute phase response in fish. In our study, we provided direct evidence that Vtg increased in seminal plasma, which suggests its important role in the protection of the male reproductive system against bacterial infection.

4.9. Protein with decreased abundance following infection

Interestingly, bacterial infection caused alterations in lysine-rich (H1, H2A, H2B) and arginine-rich (H4) histones, which in addition to their role in DNA condensation, can also be present in the extracellular space, where they serve host defense functions. Extracellular histones and their fragments act as antimicrobial peptides in the innate immune response in fish and directly kill both gram-positive and gram-negative bacteria, including *Aeromonas,* through destruction and penetration of the cell membrane and binding to bacterial DNA [72–74]. Histones have been identified in seminal plasma of different fish species, including carp [20,75]. In the present study, we provided direct evidence that seminal plasma histones are indeed altered by bacterial infection in testis. Downregulation of histones in seminal plasma following infection may result from bacterial developed mechanisms to resist killing by histones, such as binding histones to bacterial surface receptors and secretion of proteases capable of degrading histones [76] or suppression of histone expression observed in chronically stressed fish [77].

Among proteins decreasing in abundance following infection, we found proteins associated with translation (EEF1A1, RPS6KA1, RPS23), cell proliferation and development (COPS2). Moreover, we observed downregulation of CCDC62, which is involved in the maintenance of normal spermiogenesis and plays a potential role in acrosome formation, cytoplasm removal, tail stretching, and motility facilitation in mammals [78]. Their changes can suggest more extensive spermatogenesis and spermiogenesis, when round spermatids undergo a number of characteristic changes, including DNA compaction and the removal of ribosomes during cytoplasmic extrusion, in control fish as opposed to infected fish.

4.10. Bacterial proteins in seminal plasma

Our proteomic analysis indicated the presence of variable bacterial proteins in carp seminal plasma following infection, such as proteins associated with transcription (hupB, rpoA), translation including

elongation factors (tuf1, fusA) and 30S and 50S ribosomal proteins (rpsJ, rplL), lipid metabolism (fadB), transport (ABC and TRAP transporters, atpA) as well as outer membrane proteins (omph, lpp, Pal), chaperone (GroEL), and multiple enzymes (aspA, eno, cysK, fbaA, ahpC, pkc, ipdA) recognized also as actively secreted [79]. Among bacterial proteins, we identified canonical bacterial virulence factors described for *Aeromonas,* including outer membrane protein H with porin activity acting like lectin-type adhesins, which bind the bacteria to carbohydrate-rich surfaces such as erythrocytes, and extracellular enzymes, serine protease aspA (also known as the subtilase family) and enolase, contributing to bacterial dissemination. The identification of bacterial proteins in seminal plasma provided further direct evidence that *A. salmonicida* and/or its components cross the blood-testis barrier and enter the testis, leading to local infection/inflammation, which was previously indicated by an increase in proinflammatory gene expression and detection of *A. salmonicida* 16S rRNA expression [19].

4.11. Relationship between mRNA and protein levels of altered seminal proteins

Analysis of immune tissues were performed to provide some insights into relationship between the level of upregulated proteins in seminal plasma and their synthesis in immune tissues in order to evaluate possible origin of these proteins in seminal plasma. The increase in seminal protein abundance following infection may result from their synthesis and secretion in the testis (*ckm, c7, c3b, fn1*) and/or their origin from blood through the blood-testis barrier since mRNA expression of these proteins was also upregulated in hematopoietic organs. The blood proteins are synthetized by the liver as well as by head kidney and spleen, a major lymphoid organ in teleost fish [80]. We confirmed the positive relationship between mRNA and seminal protein changes for DPEP, EPDR1, EPX, NAGA, PBSP, CETP, FGA, CP in the liver, EPX, CETP, FGA, CP in the spleen and DPEP, A2M3, CETP in the kidney, which suggests that these organs can be the main source of protein changes in seminal plasma, these proteins can reach plasma via blood-testis barrier from blood to testis. Conversely, we observed a discrepancy between the protein and transcript levels of HAPLN1, C3a3, APOA2, APOA1, and A2M3 in the testis and vitellogenin in the liver because negative relationships were observed. A weak correlation between proteomic and transcriptomic analysis following bacterial and viral infection has been observed in previous studies in fish indicating both the same expression pattern and opposite expression trends for differentially abundant proteins and genes [81–83]. The uncoupling between the level of mRNA and protein can result from various factors, such as weak ribosome binding sites, codon usage bias, half-life differences between protein and mRNA, protein turnover, peptide sharing among isoforms, and posttranslational modification (PTM) variants [84]. The lack of such correlation can be related to complex relationship between the level of these proteins in seminal plasma and their synthesis in four different tissues. Overall, our results indicated that infection caused profound changes in expression of several genes in the testis as well as in hematopoietic organs, which likely contribute to changes in seminal proteins.

Teleost fish species possess multiple forms of the complement C3, five C3 isoforms are present in common carp serum [85]. In our experiment we observed the increase in protein abundance of two different isoforms of complement C3 identified as complement C3-like (*c3b,* KTF92880.1) and complement C3a, partial (*c3a3*, KTG40750.1) in seminal plasma following bacterial infection. The first C3 isoform increased both at protein and mRNA level in all analyzed tissue while the protein changes of the second C3 isoform was inconsistent with mRNA level which was unchanged in liver, kidney and spleen and down-regulated in testis. The latter C3 isoform increased in abundance in seminal plasma was identified as a fragment of C3 probably derived from protein autolysis, due to characteristics of the complement C3 proteins [86] and therefore we did not observed the relationship

between protein and mRNA levels.

We would like to mention that information on gene expression changes in response to *A. salmonicida* infection in cyprinids is limited. Similarly to our study upregulation of complement component genes was observed in zebrafish [87]. Recently Ling et al. [28] demonstrated changes in transcriptome of kidney in crucian carp infected by A. salmonicida indicating the activation of specific immune pathways after infection. Unfortunately differentially expressed genes are not listed in this paper which did not allow us to discuss our results of gene expression with data of other results. Most studies of *A. salmonicida* infection have examined changes in gene expression in salmonid species and demonstrated induction of genes associated with complement and coagulation cascade (*c3b, c7, fga*) among others in liver, spleen and kidney [88–92]. However, gene expression varies markedly among different species and even among different tissues following *A. salmonicida* infection. It should be underlined that our study is the first indicating changes of expression of some genes (upregulation of *c3b, c7a, fn, fga, ckmb* and downregulation of *hapln1, c3a3, a2m3, apoa1, apo14*) in testis which indicate that infection of *A. salmonicida* can influence male reproductive system. Further studies are necessary to determine if bacterial infection has an impact on male reproductive functions.

In conclusion, our results provide new insights into the molecular mechanisms that regulate the immune response to bacterial infection in the carp reproductive tract. *A. salmonidica* infection induced humoral and cellular innate immune response mechanisms in carp testis, including eosinophil degranulation, acute phase signaling, complement and coagulation cascade, inflammation, lipoprotein remodeling and lipid metabolism, DPEP1-and FN1-mediated adhesion, remodeling of ECM, detoxification, energy metabolism, calcium signaling and calcium buffering system. Moreover, infection led to the induction of vitellogenin production in seminal plasma. However, further studies are necessary to determine the specific roles of the described proteins in the immune response against bacteria in the testis as well as their involvement in protection of reproductive tract or induction of gamete and tissue damage. These proteins combined with bacterial proteins identified in seminal plasma may serve as valuable markers of infection in the testis of fish.

CRediT authorship contribution statement

Mariola A. Dietrich: Conceptualization, Investigation, Data curation, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing. **Mikołaj Adamek:** Investigation, Methodology, Data curation, Formal analysis, Writing – review & editing. **Felix Teitge:** Investigation, Data curation. **Lukas Teich:** Investigation, Data curation. **Verena Jung-Schroers:** Investigation, Data curation. **Agata Malinowska:** Investigation, Data curation. **Bianka Świderska:** Investigation, Data curation. **Krzysztof Rakus:** Investigation, Methodology, Data curation. **Natalia Kodzik:** Investigation, Data curation. **Magdalena Chadzińska:** Formal analysis. **Halina Karol:** Investigation. **Ewa Liszewska:** Investigation. **Andrzej Ciereszko:** Conceptualization, Writing – original draft, Writing – review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.07.019.

References

[1] P. Wang, J. Li, T.T. He, N. Li, Z.L. Mo, P. Nie, H.X. Xie, Pathogenic characterization of *Aeromonas salmonicida* subsp. masoucida turbot isolate from China, J. Fish. Dis. 10 (2010) 1145–1154, https://doi.org/10.1111/jfd.13224.

[2] S. Menanteau-Ledouble, G. Kumar, M. Saleh, M. El-Matbouli, Aeromonas salmonicida: updates on an old acquaintance, Dis. Aquat. Org. 120 (2016) 49–68, https://doi.org/10.3354/dao03006.

[3] B. Austin, D.A. Austin, *Aeromonadaceae* representatives (motile Aeromonas), in: B. Austin, D.A. Austi (Eds.), Bacterial Fish Pathogens, Springer, Cham, Edinburgh, 2016, pp. 161–214.

[4] F.A.O. Yearbook, Fishery and aquaculture statistics. https://www.fao.org/fishery/static/Yearbook/YB2019_USBcard/index.htm, 2021.

[5] D. Evenberg, P. Graaff, W. Fleuren, W.B. Muiswinkel, Blood changes in carp (*Cyprinus carpio*) induced by ulcerative *Aeromonas salmonicida* infections, Vet. Immunol. Immunopathol. 12 (1986) 321–330, https://doi.org/10.1016/0165-2427(86)90136-4.

[6] H. Kodama, H. Nakamura, M. Kashima, T. Iwasaki, H. Togase, Protection against atypical *Aeromonas salmonicida* infection in common carp, *Cyprinus carpio* L., by oral administration of a mixed microbial culture of *Lactobacillus paracasei, Pichia membranifaciens* and *Saccharomyces cereviciae*, J. Vet. Med. Sci. 73 (2011) 1319–1325, https://doi.org/10.1292/jvms.11-0180.

[7] R.A.H. Bhat, D. Thakuria, M.K. Dubey, R.S. Tandel, P. Sharma, V.C. Khangembam, P. Dash, G. Tripathi, D. Sarma D, Lethal dose and histopathological alterations induced by *Aeromonas salmonicida* in experimentally challenged common carp, *Cyprinus carpio*, Microb. Pathog. 158 (2021) 105–110, https://doi.org/10.1016/j.micpath.2021.105110.

[8] A. Lü, X. Hu, Y. Wang, X. Shen, X. Li, A. Zhu, J. Tian, Q. Ming, Z. Feng, iTRAQ analysis of gill proteins from the zebrafish (*Danio rerio*) infected with *Aeromonas hdrophila*, Fish Shellfish Immunol. 36 (2014) 229–239, https://doi.org/10.1016/j.fsi.2013.11.007.

[9] M. Long, J. Zhao, T. Li, C. Tafalla, Q. Zhang, X. Wang, X. Gong, Z. Shen, A. Li, Transcriptomic and proteomic analyses of splenic immune mechanisms of rainbow trout (*Oncorhynchus mykiss*) infected by *Aeromonas salmonicida* subsp. *Salmonicida*, J. Proteomics 122 (2015) 41–54, https://doi.org/10.1016/j.jprot.2015.03.031.

[10] G. Di, H. Li, C. Zhang, Y. Zhao, C. Zhou, S. Naeem, L. Li, X. Kong, Label-free proteomic analysis of intestinal mucosa proteins in common carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*, Fish Shellfish Immunol. 66 (2017) 11–25, https://doi.org/10.1016/j.fsi.2017.04.025.

[11] P.F. Liu, Y. Du, L. Meng, X. Li, Y. Liu Y, Proteomic analysis in kidneys of Atlantic salmon infected with *Aeromonas salmonicida* by iTRAQ, Dev. Comp. Immunol. 72 (2017) 140–153, https://doi.org/10.1016/j.dci.2017.02.013.

[12] L. Wang, C. Shao, W. Xu, Q. Zhou, N. Wang, S. Chen, Proteome profiling reveals immune responses in Japanese flounder (*Paralichthys olivaceus*) infected with Edwardsiella tarda by iTRAQ analysis, Fish Shellfish Immunol. 66 (2017) 325–333, https://doi.org/10.1016/j.fsi.2017.05.022.

[13] D.R. Causey, M.A.N. Pohl, D.A. Stead, S.A.M. Martin, C.J. Secombes, D.J. Macqueen, High-throughput proteomic profiling of the fish liver following bacterial infection, BMC Genom. 19 (2018) 719, https://doi.org/10.1186/s12864-018-5092-0.

[14] S. Bhushan, M.S. Theas, V.A. Guazzone, P. Jacobo, M. Wang, M. Fijak, A. Meinhardt, L. Lustig, Immune cell subtypes and their function in the testis, Front. Immunol. 11 (2020), 583304, https://doi.org/10.3389/fimmu.2020.583304.

[15] E. Chaves-Pozo, J. Montero, A. Cuesta, C. Tafalla, Viral hemorrhagic septicemia and infectious pancreatic necrosis viruses replicate differently in rainbow trout gonad and induce different chemokine transcription profiles, Dev. Comp. Immunol. 34 (2010) 648–658, https://doi.org/10.1016/j.dci.2010.01.009.

[16] Y. Valero, A. García-Alcázar, M.Á. Esteban, A. Cuesta, E. Chaves-Pozo, Antimicrobial response is increased in the testis of European sea bass, but not in gilthead seabream, upon nodavirus infection, Fish Shellfish Immunol. 44 (2015) 203–213, https://doi.org/10.1016/j.fsi.2015.02.015.

[17] A. Ciereszko, T. Wlasow, S. Dobosz, K. Goryczko, J. Glogowski, Blood cells in rainbow trout *Oncorhynchus mykiss* milt: relation to milt collection method and sampling period, Theriogenology 62 (2004) 1353–1364, https://doi.org/10.1016/j.theriogenology.2004.02.003.

[18] E. Chaves-Pozo, P. Pelegrín, V. Mulero, J. Meseguer, A. García Ayala, A role for acidophilic granulocytes in the testis of the gilthead seabream (*Sparus aurata* L., Teleostei), J. Endocrinol. 179 (2) (2011) 165–174, https://doi.org/10.1677/joe.0.1790165.

[19] M.A. Dietrich, M. Adamek, V. Jung-Schroers, KŁ. Rakus, M. Chadzińska, A. Hejmej, P. Hliwa, B. Bilińska, H. Karol, A. Ciereszko, Characterization of carp seminal plasma Wap65-2 and its participation in the testicular immune response and temperature acclimation, Vet. Res. 51 (2020) 142, https://doi.org/10.1186/s13567-020-00858-x.

[20] M.A. Dietrich, G.J. Arnold, J. Nynca, T. Fröhlich, K. Otte, A. Ciereszko, Characterization of carp seminal plasma proteome in relation to blood plasma, J. Proteonomics 98 (2014) 218–232, https://doi.org/10.1016/j.jprot.2014.01.005.

[21] C.J. Bayne, L. Gerwick, The acute phase response and innate immunity of fish, Dev. Comp. Immunol. 25 (2001) 725–743, https://doi.org/10.1016/S0145-305X(01)00033-7.

[22] M. Adamek, H. Syakuri, S. Harris, KŁ. Rakus, G. Brogden, M. Matras, I. Irnazarow, D. Steinhagen, Cyprinid herpesvirus 3 infection disrupts the skin barrier of common carp (*Cyprinus carpio* L.), Vet. Microbiol. 162 (2013), https://doi.org/10.1016/j.vetmic.2012.10.033, 456-447.

[23] M.A. Dietrich, S. Judycka, D. Żarski, A. Malinowska, B. Świderska, K. Palińska-Żarska, M. Błażejewski, A. Ciereszko, Proteomic analysis of pikeperch seminal plasma provides novel insight into the testicular development of domesticated fish stocks, Animal 15 (2021), 100279, https://doi.org/10.1016/j.animal.2021.100279.

[24] A. Malinowska, M. Kistowski, M. Bakun, T. Rubel, M. Tkaczyk, J. Mierzejewska, M. Dadlez, Diffprot - software for non-parametric statistical analysis of differential proteomics data, J. Proteomics 75 (2012) 4062–4073, https://doi.org/10.1016/j.jprot.2012.05.030.

[25] J.E. Elias, W. Haas, B.K. Faherty, S.P. Gygi, Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations, Nat. Methods 2 (2005) 667–675, https://doi.org/10.1038/nmeth785.

[26] M. Bakun, J. Karczmarski, J. Poznanski, T. Rubel, M. Rozga, A. Malinowska, D. Sands, E. Hennig, J. Oledzki, J. Ostrowski, M. Dadlez, An integrated LC-ESI-MS platform for quantitation of serum peptide ladders. Application for colon carcinoma study, Proteomic, Clin. Appl. 3 (2009) 932–946, https://doi.org/10.1002/prca.200800111.

[27] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)), Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[28] X.D. Ling, W.T. Dong, Y. Zhang, X. Qian, W.D. Zhang, W.H. He, X.X. Zhao, J.X. Liu, Comparative transcriptomics and histopathological analysis of crucian carp infection by atypical *Aeromonas salmonicida*, Fish Shellfish Immunol. 94 (2019) 94–307, https://doi.org/10.1016/j.fsi.2019.09.006.

[29] M. Sakai, J. Hikima, T. Kono, Fish cytokines: current research and applications, Fish. Sci. 87 (2021) 1–9, https://doi.org/10.1007/s12562-020-01476-4.

[30] Z. Gan, S.N. Chen, B. Huang, J. Zou, P Fish type I and type II interferons: composition, receptor usage, production and function, Rev. Aquacult. 12 (2019) 773–804, https://doi.org/10.1111/raq.12349.

[31] M.I. Concha, V.J. Smith, K. Castro, A. Bastías, A. Romero, R.J. Amthauer, Apolipoproteins A-I and A-II are potentially important effectors of innate immunity in the teleost fish *Cyprinus carpio*, Eur. J. Biochem. 271 (2004) 2984–2990, https://doi.org/10.1111/j.1432-1033.2004.04228.x.

[32] M.A. Dietrich, M. Adamek, B. Bilińska, 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, https://doi.org/10.1016/j.fsi.2014.09.020.

[33] P. Yang, N.P. Skiba, G.M. Tewkesbury, V.M. Treboschi, P. Baciu, G.J. Jaffe, Complement-mediated regulation of apolipoprotein E in cultured human RPE cells, Invest. Ophthalmol. Vis. Sci. 58 (2017) 3073–3085, https://doi.org/10.1167/iovs.16-20083.

[34] F.L. Li, C.P. Lu, Purification and characterization of alpha2-macroglobulin from grass carp *Ctenopharyngodon idellus*: cloning a segment of the corresponding gene, Fish Shellfish Immunol. 20 (2006) 474–481, https://doi.org/10.1016/j.fsi.2005.06.006.

[35] Y. Xiong, C. Dan, F. Ren, Z. Su, Y. Zhang, J. Mei, Proteomic profiling of yellow catfish (*Pelteobagrus fulvidraco*) skin mucus identifies differentially-expressed proteins in response to *Edwardsiella ictaluri* infection, Fish Shellfish Immunol. 100 (2020) 98–108, https://doi.org/10.1016/j.fsi.2020.02.059.

[36] I. Charlie-Silva, A. Klein, J.M.M. Gomes, E.J.R. Prado, A.C. Moraes, S.F. Eto, D.C. Fernandes, J.J. Fagliari, J.D.C. Junior, C. Lima, M. Lopes-Ferreira, K. Conceição, W.G. Manrique, M.A.A. Belo, Acute-phase proteins during inflammatory reaction by bacterial infection: fish-model, Sci. Rep. 9 (2019) 4776, https://doi.org/10.1038/s41598-019-41312-z.

[37] M. Amaya-Montoya, J.A. Pinzón-Cortés, L.S. Silva-Bermúdez, D. Ruiz-Manco, M.C. Pérez-Matos, M.A. Jiménez-Mora, C.O. Mendivil, ApoE and apoC-III-defined HDL subtypes: a descriptive study of their lecithin cholesterol acyl transferase and cholesteryl ester transfer protein content and activity, Lipids Health Dis. 19 (2020) 106, https://doi.org/10.1186/s12944-020-01291-x.

[38] I. Hardardóttir, C. Grünfeld, K.R. Feingold, Effects of endotoxin and cytokines on lipid metabolism, Curr. Opin. Lipidol. 5 (1994) 207–215, https://doi.org/10.1097/00041433-199405030-00008.

[39] O. Shimokawa, H. Nakayama, Inactivation of penicillin-induced staphylococcal L-forms by human serum high density lipoprotein, FEMS Microbiol. Lett. 156 (1997) 113–117, https://doi.org/10.1111/j.1574-6968.1997.tb12714.x.

[40] M.I. Concha, S. Molina, C. Oyarzún C, J. Villanueva, R. Amthauer, Local expression of apolipoprotein A-I gene and a possible role for HDL in primary defence in the carp skin, Fish Shellfish Immunol. 14 (2003) 259–273, https://doi.org/10.1006/fsim.2002.0435.

[41] S.R. Choudhury, L. Babes, J.J. Rahn, B.Y. Ahn, K.R. Goring, J.C. King, A. Lau, B. Petri, X. Hao, A.K. Chojnacki, A. Thanabalasuriar, E.F. McAvoy, S. Tabariès, C. Schraeder, K.D. Patel, P.M. Siegel, K.A. Kopciuk, D.C. Schriemer, D.A. Muruve, M.M. Kelly, B.G. Yipp, P. Kubes, S.M. Robbins, D.L. Senger, Dipeptidase-1 is an adhesion receptor for neutrophil recruitment in lungs and liver, Cell 178 (2019) 1205–1221, https://doi.org/10.1016/j.cell.2019.07.017, e17.

[42] B. Henderson, S. Nair, J. Pallas, M.A. Williams, Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins, FEMS Microbiol. Rev. 35 (2011) 147–200, https://doi.org/10.1111/j.1574-6976.2010.00243.x.

[43] A.I. Bachir, A.R. Horwitz, W.J. Nelson, J.M. Bianchini, Actin-based adhesion modules mediate cell interactions with the extracellular matrix and neighboring cells, Cold Spring Harbor Perspect. Biol. 9 (7) (2017) a023234, https://doi.org/10.1101/cshperspect.a023234.

[44] D.J. Vaca, A. Thibau, M. Schütz, P. Kraiczy, L. Happonen, J. Malmström, V.A. J. Kempf, Interaction with the host: the role of fibronectin and extracellular matrix proteins in the adhesion of Gram-negative bacteria, Med. Microbiol. Immunol. 209 (2020) 277–299, https://doi.org/10.1007/s00430-019-00644-3.

[45] H. Frey, N. Schroeder, T. Manon-Jensen, R.V. Iozzo, L. Schaefer, Biological interplay between proteoglycans and their innate immune receptors in inflammation, FEBS J. 280 (10) (2013) 2165–2179, https://doi.org/10.1111/febs.12145.

[46] A.C. Petrey, C.A. Motte, Hyaluronan, a crucial regulator of inflammation, Front. Immunol. 5 (2014) 101, https://doi.org/10.3389/fimmu.2014.00101.

[47] S.J. Tang, K.H. Sun, G.H. Sun, G. Lin, W.W. Lin, M.J. Chuang, Cold-induced ependymin expression in zebrafish and carp brain: implications for cold acclimation, FEBS Lett. 459 (1997) 95–99, https://doi.org/10.1016/s0014-5793(99)01229-6.

[48] C. McDougall, M.J. Hammond, S.C. Dailey, I.M.L. Somorjai, S.F. Cummins, B. M Degnan, The evolution of ependymin-related proteins, BMC Evol. Biol. 18 (2018) 182, https://doi.org/10.1186/s12862-018-1306-y.

[49] N. Yamamoto, M. Urade, Pathogenic significance of alpha-N-acetylgalactosaminidase activity found in the hemagglutinin of influenza virus, Microb. Infect. 7 (2005) 674–681, https://doi.org/10.1016/j.micinf.2005.01.015.

[50] N. Yamamoto, Pathogenic significance of alpha-N-acetylgalactosaminidase activity found in the envelope glycoprotein gp160 of human immunodeficiency virus Type 1, AIDS Res. Hum. Retrovir. 22 (2006) 262–271, https://doi.org/10.1089/aid.2006.22.262.

[51] R.C. Allen, M.L. Henery, J.C. Allen, R.J. Hawks, J.T. Stephens, Myeloperoxidase and eosinophil peroxidase inhibit endotoxin activity and increase mouse survival in a lipopolysaccharide lethal dose 90% Model, J. Immunol. Res. (2019), 4783018, https://doi.org/10.1155/2019/4783018, 2019.

[52] G. Kumar, S. Menanteau-Ledouble, M. Saleh, M. El-Matbouli, *Yersinia ruckeri*, the causative agent of enteric redmouth disease in fish, Vet. Res. 46 (2015) 103, https://doi.org/0.1186/s13567-015-0238.

[53] K.M. Choi, M.S. Joo, G. Kang, W.S. Woo, K.H. Kim, S.H. Jeong, M.Y. Son, D.H. Kim, C.I. Park, First report of eosinophil peroxidase in starry flounder (*Platichthys stellatus*): gene identification and gene expression profiling, Fish Shellfish Immunol. 118 (2021) 155–159, https://doi.org/10.1016/j.fsi.2021.08.021.

[54] K.M. Balla, G. Lugo-Villarino, J.M. Spitsbergen, D.L. Stachura, Y. Hu, K. Bañuelos, O. Romo-Fewell, R.V. Aroian, D. Traver, Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants, Blood 116 (2010) 3944–3954, https://doi.org/10.1182/blood-2010-03-267419.

[55] M. Noia, F. Fontenla-Iglesias, A. Valle, V. Blanco-Abad, J.M. Leiro, J. Lamas J, Characterization of the turbot *Scophthalmus maximus* (L.) myeloperoxidase. An insight into the evolution of vertebrate peroxidases, Dev. Comp. Immunol. 118 (2021) 103993, https://doi.org/10.1016/j.dci.2021.103993.

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

[57] T.Y. Magarlamov, D.I. Melnikova, A.V. Chernyshev, Tetrodotoxin-producing bacteria: detection, distribution and migration of the toxin in aquatic systems, Toxins 9 (2017) 166, https://doi.org/10.3390/toxins9050166.

[58] R.M. Johnson, A.C. Olatunde, L.N. Woodie, M.W. Greene, E.H. Schwartz, The systemic and cellular metabolic phenotype of infection and immune response to listeria monocytogenes, Front. Immunol. 11 (2021) 614697. https://doi.org/10.3389/fimmu.2020.614697.

[59] D.J. Kominsky, E.L. Campbell, S.P. Colgan, Metabolic shifts in immunity and inflammation, J. Immunol. 184 (2010) 4062–4068, https://doi.org/10.4049/jimmunol.0903002.

[60] D. Kitzenberg, S.P. Colgan, L.E. Glover, Creatine kinase in ischemic and inflammatory disorders, Clin. Transl. Med. 5 (2016) 31, https://doi.org/10.1186/s40169-016-0114-5.

[61] Y.Y. Gao, D.F. Zhang, H. Li, R. Liu, Z.H. Zhuang, Q.F. Li, S.Y. Wang, X.X. Peng, Proteomic approach for caudal trauma-induced acute phase proteins reveals that creatine kinase is a key acute phase protein in amphioxus humoral fluid, J. Proteome Res. 6 (2007) 4321–4329, https://doi.org/10.1021/pr070504x.

[62] N. Tian, L. Hu, Y. Lu, L. Tong, M. Feng, Q. Liu, Y. Li, Y. Zhu, L. Wu, Y. Ji, P. Zhang, T. Xu, X. Tong, TKT maintains intestinal ATP production and inhibits apoptosis-induced colitis, Cell Death Dis. 12 (10) (2021) 853, https://doi.org/10.1038/s41419-021-04142-4.

[63] E.L. Pearce, E.J. Pearce, Metabolic pathways in immune cell activation and quiescence, Immunity 38 (2013) 633–643, https://doi.org/10.1016/j.immuni.2013.04.005.

[64] S. Fu, M. Ding, Q. Liang, Y. Yang, M. Chen, X. Wei, A. Wang, S. Liao, J. Ye, The key differentially expressed genes and proteins related to immune response in the spleen of pufferfish (*Takifugu obscura*) infected by *Aeromonas hydrophila*, Fish Shellfish Immunol. 91 (2019) 1–11, https://doi.org/10.1016/j.fsi.2019.05.016.

[65] M.A. Dietrich, J. Nynca, B. Bilińska, J. Kuba, M. Kotula-Balak, H. Karol, A. Ciereszko, Identification of parvalbumin-like protein as a major protein of common carp (*Cyprinus carpio* L) spermatozoa which appears during final stage of spermatogenesis, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 157 (2010) 220–227, https://doi.org/10.1016/j.cbpb.2010.06.007.

[66] M.A. Dietrich, B. Westfalewicz, P. Jurecka, I. Irnazarow, A. Ciereszko, Isolation, characterisation and cDNA sequencing of a new form of parvalbumin from carp semen, Reprod. Fertil. Dev. 26 (2014) 1117–1128, https://doi.org/10.1071/RD13181.

[67] A.R. Porta, E. Bettini, O.I. Buiakova, H. Baker, W. Danho, F.T. Margolis, Molecular cloning of ictacalcin: a novel calcium-binding protein from the channel catfish, *Ictalurus punctatus*, Brain. Res. Mol. Brain. Res. 41 (1996) 81–89, https://doi.org/10.1016/0169-328x(96)00069-1.

[68] L.J. Mills, R.E. Gutjahr-Gobell, D.B. Horowitz, N.D. Denslow, M.C. Chow, G. E. Zaroogian, Relationship between reproductive success and male plasma vitellogenin concentrations in cunner, *Tautogolabrus adspersus*, Environ. Health Perspect. 111 (2003) 93–100, https://doi.org/10.1289/ehp.5531.

[69] Q.H. Liu, S.C. Zhang, Z.J. Li, C.R. Gao, Characterization of a pattern recognition molecule vitellogenin from carp (*Cyprinus carpio*), Immunobiology 214 (2009) 257–267, https://doi.org/10.1016/j.imbio.2008.10.003.

[70] X. Shi, S. Zhang, Q. Pang, Vitellogenin is a novel player in defense reactions, Fish Shellfish Immunol. 20 (2006) 769–772, https://doi.org/10.1016/j.fsi.2005.09.005.

[71] Z. Tong, L. Li, R. Pawar, S. Zhang, Vitellogenin is an acute phase protein with bacterial-binding and inhibiting activities, Immunobiology 215 (2010) 898–902, https://doi.org/10.1016/j.imbio.2009.10.001.

[72] J.M. Fernandes, G.D. Kemp, M.G. Molle, V.J. Smith, Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*, Biochem. J. 368 (2002) 611–620, https://doi.org/10.1042/BJ20020980.

[73] Y. Valero, M. Arizcun, M.Á. Esteban, A. Cuesta, E. Chaves-Pozo, Transcription of histones H1 and H2B is regulated by several immune stimuli in gilthead seabream and European sea bass, Fish Shellfish Immunol. 57 (2016) 107–115, https://doi.org/10.1016/j.fsi.2016.08.019.

[74] B. Fu, H. Lin, T. Ramesh Pavase, N. Mi, J. SuI, Extraction, identification, modification, and antibacterial activity of histone from immature testis of Atlantic salmon, Mar. Drugs 18 (2020) 133, https://doi.org/10.3390/md18030133.

[75] M.A. Dietrich, J. Nynca, B. Westfalewicz, H. Karol, A. Ciereszko, Identification of calcium-binding proteins in fish seminal plasma, Fish Physiol. Biochem. 37 (2011) 447–452, https://doi.org/10.1007/s10695-010-9445-0.

[76] M. Hoeksema, M. van Eijk, H.P. Haagsman, K.L. Hartshorn, Histones as mediators of host defense, inflammation and thrombosis, Future, Microbiol. 11 (2016) 441–453, https://doi.org/10.2217/fmb.15.151.

[77] D.W. Robinette, E.J. Noga, Histone-like protein: a novel method for measuring stress in fish, Dis. Aquat. Org. 44 (2001) 97–107, https://doi.org/10.3354/dao044097.

[78] Y. Li, C. Li, S. Lin, B. Yang, W. Huang, H. Wu, Y. Chen, L. Yang, M. Luo, H. Guo, J. Chen, T. Wang, Q. Ma, Y. Gu, L. Mou, Z. Jiang, J. Xia, Y. Gui, A nonsense mutation in Ccdc62 gene is responsible for spermiogenesis defects and male infertility in repro29/repro29 mice, Biol. Reprod. 96 (2017) 587–597, https://doi.org/10.1095/biolreprod.116.141408.

[79] P. Vanden Bergh, M. Heller, S. Braga-Lagache, J. Frey, The Aeromonas salmonicida subsp. salmonicida exoproteome: determination of the complete repertoire of Type-Three Secretion System effectors and identification of other virulence factors, Proteome Sci. 11 (2013) 42, https://doi.org/10.1186/1477-5956-11-42.

[80] G. Kumar, K. Hummel, K. Noebauer, T.J. Welch, E. Razzazi-Fazeli, M. El-Matbouli, Proteome analysis reveals a role of rainbow trout lymphoid organs during *Yersinia ruckeri* infection process, Sci. Rep. 8 (2018), 13998, https://doi.org/10.1038/s41598-018-31982-6.

[81] J.N. Li, Y.T. Zhao, S.L. Cao, H. Wang, J.J. Zhang, Integrated transcriptomic and proteomic analyses of grass carp intestines after vaccination with a double-targeted DNA vaccine of *Vibrio mimicus*, Fish Shellfish Immunol. 98 (2020) 641–652, https://doi.org/10.1016/j.fsi.2019.10.045.

[82] L. Qi, Y. Chen, K. Shi, H. Ma, S. Wei, Z. Sha, Combining of transcriptomic and proteomic data to mine immune-related genes and proteins in the liver of *Cynoglossus semilaevis* challenged with *Vibrio anguillarum*, Comp. Biochem. Physiol., Part D: Genomics Proteomics 39 (2021), 100864, https://doi.org/10.1016/j.cbd.2021.100864.

[83] Y.Y. Wang, Y. Zhou, H.C. Fu, H.Z. Huang, Z. Li, R.M. Jin, X.Z. Fu, N.Q. Li, Transcriptomic and proteomic analyses of the immune mechanism in pathogenetic and resistant Mandarin fish (*Siniperca chuatsi*) infected with ISKNV, Aquaculture 545 (2021), 737198, https://doi.org/10.1016/j.aquaculture.2021.737198.

[84] Y. Liu, A. Beyer, R. Aebersold, On the dependency of cellular protein levels on mRNA abundance, Cell 165 (2016) 535–550, https://doi.org/10.1016/j.cell.2016.03.014.

[85] M. Nakao, J. Mutsuro, R. Obo, K. Fujiki, M. Nonaka, T. Yano, Molecular cloning and protein analysis of divergent forms of the complement component C3 from a bony fish, the common carp (*Cyprinus carpio*): presence of variants lacking the catalytic histidine, Eur. J. Immunol. 30 (2000) 858–866, https://doi.org/10.1002/1521-4141(200003)30:3<858::AID-IMMU858>3.0.CO;2-M.

[86] R.B. Sim, E. Sim, Autolytic fragmentation of complement components C3 and C4 and its relationship to covalent binding activity, Ann. N. Y. Acad. Sci. 42 (1983) 259–276, https://doi.org/10.1111/j.1749-6632.1983.tb18114.x.

[87] B. Lin, S. Chen, Z. Cao, Y. Lin, D. Mo, H. Zhang, J. Gu, M. Dong, Z. Liu, A. Xu, Acute phase response in zebrafish upon *Aeromonas salmonicida* and *Staphylococcus aureus* infection: striking similarities and obvious differences with mammals, Mol. Immunol. 44 (2007) 295–301, https://doi.org/10.1016/j.molimm.2006.03.001.

[88] K.V. Ewart, J.C. Belanger, J. Williams, T. Karakach, S. Penny, S.C. Tsoi, R. C. Richards, S.E. Douglas, Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology, Dev. Comp. Immunol. 29 (2005) 333–347, https://doi.org/10.1016/j.dci.2004.08.004.

[89] M.H. Marana, A.M. Karami, J. Ødegård, S. Zuo, R.M. Jaafar, H. Mathiessen, L. von Gersdorff Jørgensen, P.W. Kania, I. Dalsgaard, T. Nielsen, K. Buchmann, Whole-

genome association study searching for QTL for *Aeromonas salmonicida* resistance in rainbow trout, Sci. Rep. 11 (2021), 17857, https://doi.org/10.1038/s41598-021-97437-7.

[90] J. Lim, S. Hong, Transcriptome analysis in the head kidney of rainbow trout (*Ocorhynchus mykiss*) immunized with a combined vaccine of formalin-inactivated *Aeromonas salmonicida* and *Vibrio anguillarum*, Vaccines 9 (2021) 1234, https://doi.org/10.3390/vaccines9111234.

[91] M. Liu, X. Yang, C. Zeng, H. Zhao, J. Li, Z. Hou, H. Wen, Transcriptional signatures of immune, neural, and endocrine functions in the brain and kidney of rainbow trout (*Oncorhynchus mykiss*) in response to *Aeromonas salmonicida* infection, Int. J. Mol. Sci. 23 (2022) 1340, https://doi.org/10.3390/ijms23031340.

[92] X. Li, Y. Liu, J. Cheng, Y. Xia, K. Fan, Y. Liu, P. Liu, Identification and expression analysis of a fibrinogen alpha chain-like gene in Atlantic salmon (*Salmo salar*), Aquac. Rep. 22 (2022), 100919, https://doi.org/10.1016/j.aqrep.2021.100919.