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Quantitative proteomic profiling of immune responses to *Ichthyophthirius multifiliis* in common carp skin mucus

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ABSTRACT

Ichthyophthirius multifiliis, a ciliated protozoan parasite, causes ichthyophthiriasis and leads to considerable economic losses to the aquaculture industry. Understanding the fish immune response and host-parasite interactions could support developing novel strategies for better disease management and control. Fish skin mucus is the first line of defence against infections through the epidermis. Yet, the common carp, *Cyprinus carpio*, protein-based defence strategies against infection with *I. multifiliis* at this barrier remain elusive. The skin mucus proteome of common carp was investigated at 1 day and 9 days post-exposure with *I. multifiliis*. Using nano-LC ESI MS/MS and statistical analysis, the abundance of 19 immune related and signal transduction proteins was found to be differentially regulated in skin mucus of common carp in response to *I. multifiliis*. The analysis revealed increased abundance values of epithelial chloride channel protein, galactose-specific lectin nattection, high choriolytic enzyme 1 (nephrosin), lysozyme C, granulin and protein-glutamine gamma-glutamyltransferase 2 in *I. multifiliis*-exposed carp skin mucus. Multiple lectins and a diverse array of distinct serpins with protease inhibitor activity were identified likely implicated in lectin pathway activation and regulation of proteolysis, indicating that these proteins contribute to the carp innate immune system and the protective properties of skin mucus. The results obtained from this proteomic analysis enables a better understanding of fish host response to parasitic infection and gives insights into the key role skin mucus plays in protecting fish against deleterious effects of *I. multifiliis*.

1. Introduction

In fish-pathogen interactions, immune responses in skin mucosal surfaces can be elicited in the host fish [1–4]. For control of *Ichthyophthirius multifiliis*, a pathogenic ciliate of freshwater fish with global distribution. Various strategies including chemotherapeutics and immunoprophylaxis have been used [5,6]. Although several chemotherapeutics have been successfully applied to treat ornamental fish in small aquaria, few could be safely used against the pathogen in farmed fish, and no effective vaccine has been produced [7,8]. Hence, a deeper understanding of host parasite interaction at the site of infection may support development of novel strategies to compete *I. multifiliis* [8].

Common carp, *Cyprinus carpio* has been shown to be a highly useful animal model, to supply important information on physiology, genetics, immunology, infection and disease [9]. An understanding of fish host-pathogen interactions can elucidate key regulators of host immune response, and pave the way for development of novel management strategies for disease control in aquaculture. At the interface between the host and the external environment, the fish skin mucus is the first line of defence against infection, and has been associated with a diversity of functions [2].

In vertebrates, the mucosal immune system provides important functions against infections. It prevents the uptake of microorganisms and foreign substances, and avoids the development of destructive immune responses against invasive pathogens [10]. The protective role of fish skin mucus is of immense economic significance, as infectious diseases limit intensive aquaculture, globally [7]. It is known that mucus secretion is increased in fish affected by one aquatic pathogen in particular, the ciliated *I. multifiliis* [11]. It has been documented that

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theronts move rapidly through the mucus on their way into the skin [12]. Theronts enter the host by moving in between two epithelial cells [13]. As this is exactly where mucus cells open to the fish surface it is most likely that the invasive stages get access to the epidermis by invading mucous cells thereby increasing mucus production upon infection [11]. It has been observed that theronts were attracted by fish mucus and chemotactically responding to serum components in mucus [14]. This suggests that mucus plays an active role in the immune response against this pathogen. Cellular and cytokine responses were studied in common carp infected with *I. multifiliis* and were reported to be comparable to the pattern observed by mechanical injuries and ascribed to penetrating wounds caused by *I. multifiliis* at infection sites [15]. Hence, we suggested that common carp mucus contains certain components (proteins) that may have a role in protecting fish and preventing excessive tissue destruction caused by the parasite during infestation. Thus, a comprehensive proteomic comparison of skin mucus from naïve (non-exposed) fish versus those exposed to *I. multifiliis* would be useful to reveal specific mucus components that have a significant role against the pathogen.

The carp skin mucus proteome was explored in response to *I. multifiliis* infection, using nano-LC ESI MS/MS, a label-free quantitative approach. Temporal modulation of the skin mucus proteome was explored at days 1 and 9 post-exposure. Multiple proteins showed different abundance values after infection with *I. multifiliis*. The results obtained from this study not only confirm previous findings but may also support uncover novel aspects of carp-ciliate interplay and can enhance our understanding of crucial mechanisms of fish immune response to parasitic infection.

2. Material and methods

2.1. Ethics statement

All experiments were approved by the Animal Experimentation Ethics Committee of Vienna University of Veterinary medicine (BMWFW-68.205/0051-WF/V/3b/2016). All Experiments were performed in accordance with relevant guidelines and regulations.

2.2. Animals and collection of skin mucus

Specific pathogen free common carp (mean length 11 ± 1 cm) were obtained from a certified Austrian hatchery and acclimatized for 2 wk under controlled laboratory conditions, and fed 1% body weight using a commercial pellet diet (Garant Aqua, Pöchlarn, Austria). Fish were subjected to virological, bacteriological and parasitological investigations to rule out the possibility of other infections prior to the *in vivo* experiment. Prior to exposure, fish were distributed between 6 aquaria, 6 fish per aquarium. There were two groups: exposed and non-exposed control. The fish were exposed to *I. multifiliis* by cohabitation with infected giant gourami (*Osphronemus goramy*) obtained from a pet store [16]. The giant gouramies were certified as free from *Aphanomyces invadans* and the *Epizootic Haematopoietic Necrosis* Virus. Examinations of the giant gourami did not reveal the incidence of parasitic infection or signs of a secondary bacterial infection. At 1 and 9 days post-exposure (dpe), common carp ($N = 3$) from each of exposed and non-exposed control groups were anaesthetised using ethyl 3-aminobenzoate methanesulfonate (Sigma, Darmstadt, Germany) (MS-222; 100 mg/L). Sterile glass slides were used to collect skin mucus from both sides (starting from head to the caudal fin base) of exposed and non-exposed control fish, while avoiding blood contamination, and excluding the ventral body surface close to the anal pore, to prevent faecal contamination. However, it should be kept in mind that the collected mucus may contain secreted or cell-associated components. Collected mucus was transferred into 1.5 mL microcentrifuge tubes, immediately placed on ice, then stored in a −80 °C freezer until further analysis.

2.3. Protein extraction

Fish mucus was solubilised using 400 µl pre-cooled denaturing lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS and 1% DTT) that contained a mammalian protease inhibitor cocktail (Sigma Aldrich, Vienna, Austria). Mucus suspensions were disrupted by sonication on ice for 5 cycles of 10 s pulse-on and 30 s pulse-off. Lysates were then incubated overnight at 4 °C. Subsequently, lysates were vortexed and then centrifuged at 18000 x g for 30 min at 4 °C and the supernatants collected. Total protein concentration of each lysate was determined colorimetrically with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) using a Pierce 660 nm Protein Assay (Pierce, Thermo Fisher Scientific) according to the manufacturer's instructions.

2.4. Protein separation and in-gel digestion

Protein samples (40 µg per lane) in biological and technical triplicates, were electrophoresed in 10% polyacrylamide separating gels then stained with silver.

GeLC-Bands were excised manually from silver-stained 1D-gels. After washing and de-staining, bands were reduced with dithiothreitol and alkylated with iodoacetamide [17]. In-gel digestion for 8 h at 37 °C was performed using trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI, USA) at a final concentration of 20 ng/µl in 50 mM aqueous ammonium bicarbonate and 5 mM $CaCl_2$ according to Shevchenko et al. [18]. Peptides were then extracted with three changes of 30 µl 5% trifluoroacidic acid (TFA) in 50% aqueous acetonitrile supported by ultrasonication for 10 min per change. Extracted peptides were dried down in a vacuum concentrator (Eppendorf, Hamburg, Germany), then redissolved in 0.1% aqueous TFA prior to LC-MS.

2.5. LC-MS/MS analysis

Peptides were separated on a nano-HPLC Ultimate 3000 RSLC system (Dionex). Samples were pre-concentrated and desalted using 5 mm Acclaim PepMap µ-Precolumns (300 µm inner diameter, 5 µm particle size, and 100 Å pore size) (Dionex). For sample loading and desalting, 2% ACN was used in ultra pure H_2O with 0.05% TFA as a mobile phase with a flow rate of 5 µl/min.

Separation of peptides was performed on a 25 cm Acclaim PepMap C18 column (75 µm inner diameter, 2 µm particle size, and 100 Å pore size) with a flow rate of 300 nl/min. The gradient started with 4% B (80% ACN with 0.1% formic acid) and increased to 35% B over 120 min, followed by a washing step with 90% B. Mobile Phase A consisted of ultra pure H_2O with 0.1% formic acid.

2.6. QTOF mass spectrometry for SWATH measurements

For mass spectrometric analysis, the LC was directly coupled to a high resolution quadrupole time of flight mass spectrometer (Triple TOF 5600 +, Sciex).

For information data independent acquisition (DIA runs), MS1 spectra were collected in the range of 400–1500 m/z with an accumulation time of 150 ms. Product ion spectra were collected in 75 windows in the range of 300–1500 m/z with a width of 7–182.5 Da depending on the density of precursor masses in the mass segment. For each window, ions were accumulated for 40 ms.

Data independent Sequential Window Acquisition of all Theoretical spectra (SWATH runs) based on MS2 quantification, was used for quantitative measurements [19]. Peptides were fragmented in 35 fixed fragmentation windows of 20 Da over 400–1100 Da with an accumulation time of 50 ms in TOF MS mode and 80 ms in product ion mode. The nano-HPLC system was operated by Chromeleon 6.8 (Dionex, USA) and the MS by Analyst Software 1.6 (Sciex, USA).

Table 1
Signal transduction related proteins of skin mucus of common carp in response to *Ichthyophthirius multifiliis*. Fold change (infected vs control) was statistically analysed in *I. multifiliis* exposed and non-exposed skin mucus samples at 1 and 9 days post-exposure (dpe). *denotes statistically significant values according to both ANOVA with FDR-adjusted p value < 0.01 and fold change < -3 or $> +3$.

Accession NCBI	Protein	Number of quantified peptides	BLASTp results	1 dpe	9 dpe	Function
XP_018953741.1	microfibril-associated glycoprotein 4-like [*Cyprinus carpio*]	5	–	**5.3***	**−1.6**	Pathogen recognition and complement activation
XP_018943256.1	galactose-specific lectin nattectin-like [*Cyprinus carpio*]	4	–	**8.4***	1.3	Pathogen recognition and complement activation
XP_018943953.1	epithelial chloride channel protein-like [*Cyprinus carpio*]	6	–	**6.3***	2.3	Pathogen recognition and sensory transduction
KTG06054.1	protein cypCar_00005920 [*Cyprinus carpio*]	4	PREDICTED: ras GTPase-activating protein-binding protein 1 isoform X1 [*Cyprinus carpio*], XP_018929740.1, 98% identity	1.4	**3.7***	Pathogen recognition and binding
KTF97319.1	cypCar_00037802 [*Cyprinus carpio*]	3	PREDICTED: interferon-induced GTP-binding protein Mx2-like [*Cyprinus carpio*], XP_018940065.1, 72% identity	2.4	**4.8***	Pathogen recognition and binding
XP_018920821.1	interferon-induced GTP-binding protein Mx-like [*Cyprinus carpio*]	4	–	2.1	**3.7***	Pathogen recognition and binding
XP_018940065.1	interferon-induced GTP-binding protein Mx2-like [*Cyprinus carpio*]	6	–	1.6	**3.3***	Pathogen recognition and binding

2.7. Data processing, quantification and statistical evaluation

Acquired raw data were processed with ProteinPilot Software version 5.0 (Sciex, USA) for re-calibration and database searches. The database consisted of NCBI entries of following taxonomies: *Cyprinus* (taxonomy id: 7961, Released 2017_02) and the common Repository of Adventitious proteins (cRAP), downloaded, http://www.thegpm.org/crap/index.html. Mass tolerances in MS mode was 0.05 Da, and 0.1 Da in MSMS mode, for the rapid re-calibration search, and 0.0011 Da in MS and 0.01 Da in MSMS mode for the final search. The following sample parameters were used: trypsin digestion, cysteine alkylation set to iodoacetamide, search effort set to rapid ID. False discovery rate analysis (FDR) was performed using the integrated tools in ProteinPilot set to < 1% on the protein level. Information-dependent acquisition (IDA) results were used to create the SWATH ion library, with the MS/MS (ALL) with SWATH Acquisition MicroApp 2.0 in PeakView 2.2 (both Sciex). Peptides were chosen based on a FDR rate < 1%, excluding shared and modified peptides. Up to 6 peptides per protein and up to 6 transitions per peptide were used. MarkerView 1.2.1 (Sciex) was used for calculation of peak areas of SWATH samples after retention time alignment and normalization using total area sums. Data were visualized using the resulting protein lists, after principal component analysis using loadings plots and score plots to get a first impression of the overall data structure, and to assess variability between technical and biological replicates.

Protein abundance was evaluated among skin mucus samples, statistically using R programming language (R Core Team 2015). Differential abundance of proteins was assessed using one-way ANOVA for each protein comparing two groups (exposed and non-exposed skin mucus) within each time point. To adjust for multiple testing, the method of Benjamini and Hochberg [20] was used to control the FDR. Differences were considered significant if adjusted p-values were smaller than the significance level of $\alpha = 0.01$. For those proteins, Tukey's honest significant difference (HSD) method was applied as post-hoc test to assess the significance of the pairwise comparisons. Protein abundance was considered differential if the adjusted p value was below α and the absolute fold change was at least three (fold change < -3 or $> +3$).

2.8. Protein-protein interaction network analysis

To determine the correlation among proteins, BLASTp searches of amino acid sequences of identified proteins were evaluated against zebrafish (*Danio rerio*) by using STRING software. Representation of the protein-protein interaction network for signal transduction and immune-related proteins was analysed at a confidence scores of 0.15 in the Databases, Experiments, Text Mining and Co-expression.

3. Results

The skin mucus proteomes of common carp that had been exposed to *I. multifiliis* were compared using a label-free quantitative proteomic approach. After principal component analysis, data were visualized with the resulting protein lists, using loadings plots and score plots to get a first impression of the overall data structure.

A total of 1233 proteins (Supplementary Table 1) were identified in skin mucus of common carp. Statistical analysis revealed a total of 44 differentially up and down-regulated proteins in skin mucus of common carp in response to *I. multifiliis*, which are involved in diverse aspects of the fish immune response: proteins with increased abundance in infected fish were mainly involved in stress and immune responses, while proteins with decreased abundance were mainly structural. Out of the 44 proteins, 19 structural and extracellular matrix proteins in addition to 6 metabolism proteins were differentially regulated in carp skin mucus in response to *I. multifiliis* infection. These proteins were not discussed in this manuscript, and were discussed separately and

Table 2
Immune related proteins of skin mucus of common carp in response to *Ichthyophthirius multifiliis*. Fold change (infected vs control) was statistically analysed in *I. multifiliis* exposed and non-exposed skin mucus samples at 1 and 9 days post-exposure (dpe). *denotes statistically significant values according to both ANOVA with FDR-adjusted p value < 0.01 and fold change < -3 or $> +3$.

NCBI accession number	Protein	Number of quantified peptides	BLASTp results	1 dpe	9 dpe	Function
Immune related proteins						
XP_018932970	granulins-like isoform X2 [*Cyprinus carpio*]	2	–	**17.0***	**4.3***	Macrophage proliferation and differentiation
XP_018930506.1	high choriolytic enzyme 1-like [*Cyprinus carpio*]	6	nephrosin precursor [*Cyprinus carpio*], AAB62737.1, 99% identity	**6.8***	1.7	Choriolytic and proteolytic activities
XP_018958317.1	lysozyme C-like [*Cyprinus carpio*]	2	–	**7.9***	2.1	Innate immunity and hydrolytic activity
KTF86954.1	cypCar_00043801 [*Cyprinus carpio*]	2	PREDICTED: leukocyte elastase inhibitor-like [*Sinocyclocheilus grahami*] XP_016104575.1, 88% identity	−1.0	**6.9***	Critical for neutrophil survival
KTF71329.1	cypCar_00049955, partial [*Cyprinus carpio*]	5	leukocyte elastase inhibitor [*Danio rerio*], XP_002665111.3, 63% identity	2.8	**10***	Critical for neutrophil survival
KTF83455.1	cypCar_00049514, partial [*Cyprinus carpio*]	4	PREDICTED: leukocyte elastase inhibitor-like [*Sinocyclocheilus grahami*] XP_016104575.1, 67% identity	2.0	**8.0***	Critical for neutrophil survival
XP_018976765.1	thioredoxin-like isoform X2 [*Cyprinus carpio*]	3	–	1.5	**4.2***	Protection against oxidative stress and infection
KTG38050.1	cypCar_00040940 [*Cyprinus carpio*]	5	protein-glutamine gamma-glutamyltransferase 5-like [*Cyprinus carpio*], XP_018944465.1, 63% identity	**4.7***	**21.3***	Key role in gamma-glutamyl cycle and maintain normal redox status
KTG03547.1	cypCar_00031278 [*Cyprinus carpio*]	5	PREDICTED: protein-glutamine gamma-glutamyltransferase 2-like isoform X2 [*Cyprinus carpio*], XP_018924257.1, 93% identity	**5.9***	**12.2***	Key role in gamma-glutamyl cycle and maintain normal redox status
XP_018981264.1	protein-glutamine gamma-glutamyltransferase 5-like [*Cyprinus carpio*]	4	–	**3.5***	**7.6***	Key role in gamma-glutamyl cycle and maintain normal redox status
KTF82164.1	cypCar_00034486 [*Cyprinus carpio*]	2	PREDICTED: protein-glutamine gamma-glutamyltransferase 5-like [Sinocyclocheilus rhinocerous], XP_016424423.1, 86% identity	**4.4***	**4.4***	Key role in gamma-glutamyl cycle and maintain normal redox status
XP_018981477.1	protein-glutamine gamma-glutamyltransferase 2-like [*Cyprinus carpio*]	5	–	**8.7***	**41.8***	Key role in gamma-glutamyl cycle and maintain normal redox status

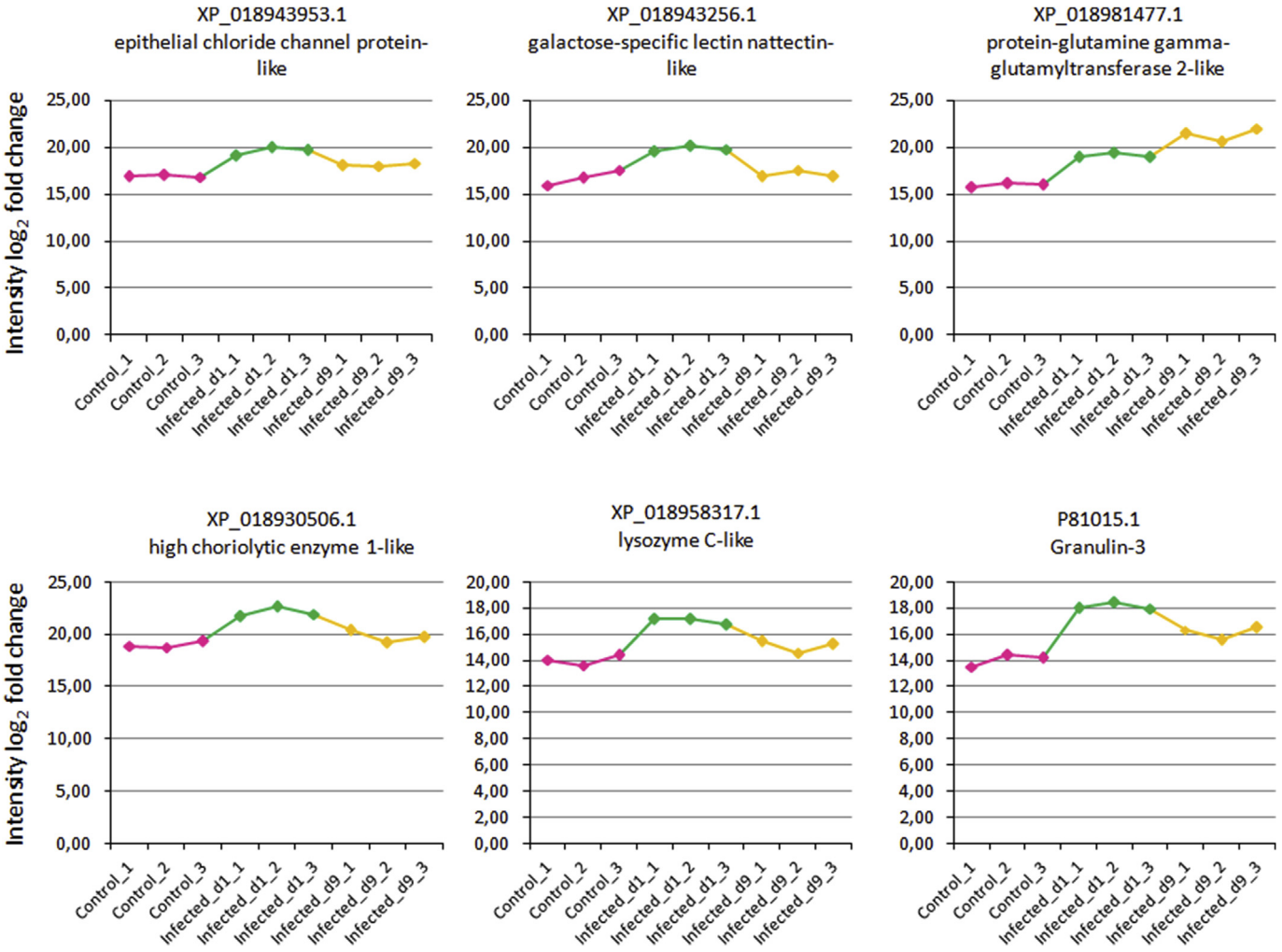

Fig. 1. Abundance plots of top candidate skin mucus proteins. Plots show fold change of proteins of carp skin mucus samples at 1 and 9 days post-exposure. The calculation based on the raw intensity values for the proteins at each time point.

reported to have a role in wound healing and tissue repair [21].

Herein, nineteen differentially regulated signal transduction and inflammatory proteins were presented (Tables 1 and 2). Some of these proteins have a recognized role in immunity such as lysozyme C and granulin, while others such as epithelial chloride channel protein and nephrosin are either involved indirectly or have potential immune functions. The abundance of top skin mucus proteins is shown in Fig. 1. Compared to control samples, infected samples show higher abundance values of epithelial chloride channel protein, lectins, nephrosin, lysozyme C, granulin, leukocyte elastase inhibitors and protein-glutamine gamma-glutamyltransferase proteins. These were significantly modulated likely to coordinate and initiate a defence strategy against *I. multifiliis*. The abundance of several anti-inflammatory proteins was increased, likely to mediate an anti-inflammatory response to reduce tissue damage provoked by *I. multifiliis*. Due to limited information on protein function and protein-protein interactions of common carp, the potential function of the identified carp skin mucus proteins is suggested after manual BLASTp analysis.

3.1. Signal transduction proteins

Following signal transduction proteins were found differentially regulated in infected carp skin mucus (Table 1): galactose-specific lectin nattectin-like, microfibril-associated glycoprotein 4-like, epithelial chloride channel protein-like, ras GTPase-activating protein-binding protein 1, and 3 interferon-induced GTP-binding protein Mx-like. These proteins likely have important roles in recognition and binding of specific pathogen-associated molecules and may activate complement which lead to opsonization, leukocyte activation, and direct pathogen killing.

3.2. Immune related proteins

The following immune-related proteins were identified: granulin, nephrosin, lysozyme C-like, 3 leukocyte elastase inhibitor-like, thioredoxin-like and 4 protein-glutamine gamma-glutamyltransferase-like. These proteins were significantly modulated to coordinate and initiate a defence strategy against *I. multifiliis*. Several anti-inflammatory proteins showed increased abundance values, apparently to mediate an anti-inflammatory response to reduce tissue damage provoked by *I. multifiliis*.

3.3. Protein-protein interaction network

Six proteins such as G3BP1, galactose-specific lectin nattectin-like protein, microfibrillar-associated protein 4, LEI, granulin and interferon-induced GTP-binding protein Mx-like were connected to each other in the network (Fig. 2). Details of protein abbreviations with node colour and edge interaction used in Fig. 2 are presented in Fig. 3. Additionally, microfibrillar-associated protein 4 and LEI showed high interaction to each other. Using protein-protein interaction analysis, lysozyme C was associated with nephrosin and thioredoxin.

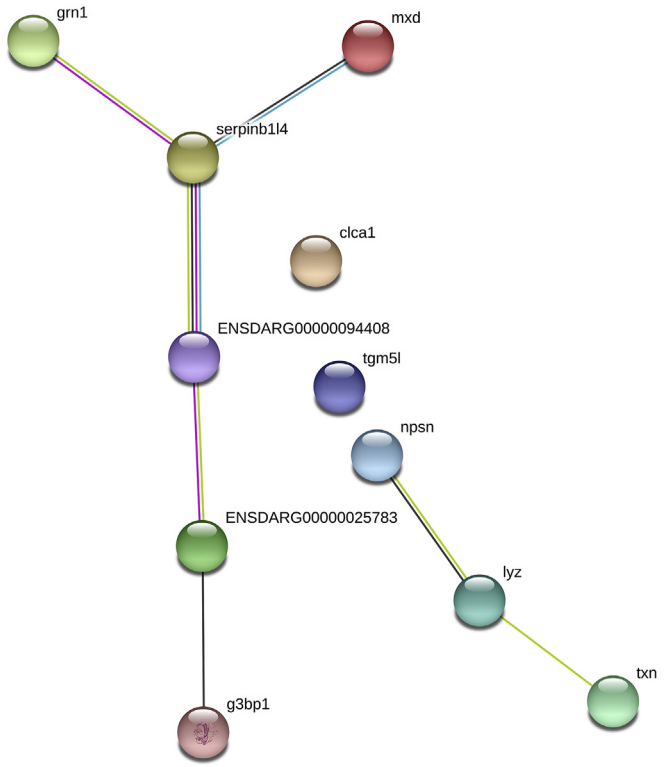

Fig. 2. The protein-protein interaction network of signal transduction and immune-related proteins of carp skin mucus. In this network, nodes are proteins, lines represent the predicted functional associations, and the number of lines represents the strength of predicted functional interactions between proteins.

The involvement of carp lectins in complement activation and recognition of *I. multifiliis* was previously suggested after *I. multifiliis* infection [3]. Besides, zebrafish nephrosin-positive cells express granulocytic markers, whereas a smaller number of these cells coexpressed the lysozyme C indicating that zebrafish nephrosin might be a granzyme in granulocytes [22].

4. Discussion

The importance of common carp skin as a site of expression of immune-related molecules against *I. multifiliis* infections has been previously investigated and several gene expression studies were carried out to investigate the modulation of fish immune response after exposure to *I. multifiliis* [3,15]. Accordingly, in this study, using proteomics, the modulation of carp proteome was investigated in skin mucus samples after infection with *I. multifiliis*, to give insight into host response against parasitic infection. The current study identifies for the first time differentially regulated immune-related proteins in common carp skin mucus after exposure to *I. multifiliis*. Due to the lack of protein databases for proteins functions and interactions in common carp, attempts were given to create a link between existing literature on fish-parasite interactions and suggested function of the proteins identified after manual BLASTp analysis. However, functional experiments are needed to determine their specific roles in carp-*I. multifiliis* interactions.

In the present study, the carp microfibrillar-associated protein 4 (MFAP4) showed increased abundance (5.3-fold) at 1 dpe. It was unclear whether or not bony fish have a functional lectin pathway linked with C3 or C4 activation until, Nakao et al., [23] showed that mannose binding lectins purified from carp serum activate C4, presenting the lectin complement pathway of carp. MFAP4 acts as pathogen receptor during the innate immune response in fish as ficolin genes in mammals [24]. As a member of the lectin family, MFAP4 has a role in recognition and binding of carbohydrates in pathogens and can activate complement which lead to opsonization, leukocyte activation, and direct pathogen killing [24]. MFAP4 has been reported to be highly expressed in a group of common carp with high survival rate and significantly lower viral loads of the CyHV-3 virus [25].

In addition to MFAP4, galactose-specific lectin nattectin-like protein showed increased abundance (8.4-fold) at 1 dpe. The involvement of carp C-type lectin molecules in the recognition of *I. multifiliis* was uncertain, although, a systemic down-regulation of a carp lectin gene expression after *I. multifiliis* infection was reported [3]. Both carp lectins; MFAP4 and galactose-specific lectin nattectin-like were significantly upregulated at 1dpe and levelled off at 9dpe which potentiates the previous observation made by Gonzalz et al., [3] that carp lectins serve as pattern recognition receptors and likely involved in *I. multifiliis* recognition. The initial increased abundance observed at 1 dpe indicates that these molecules are important during the early stages of infection likely to activate complement, opsonization and leukocytes endorsing pathogen killing.

In infected carp skin mucus, epithelial chloride channel protein, an ABCC protein showed increased abundance (6.3-fold) at 1dpe. The ABCC subfamily comprises multi-drug resistance-associated proteins that transport various substrates including drugs, endogenous compounds, and xenobiotics [26]. Intercellular transfer of proteins is a form of communication among cells that is essential for certain physiological processes. Transfer of membrane proteins is involved spreading of the glycosylphosphatidylinositol (GPI)-anchored proteins. The GPI-anchored protein, CD90, is transferred from donor cells to acceptor cells [27]. The transporter associated with antigen processing (TAP), a member of the family of ABC transporters was suggested to have an essential role in the cross-presentation of exogenous antigens [28]. The i-antigens of *Ichthyophthirius multifiliis* are GPI-anchored proteins [29]. Clustering of i-antigen in *Ichthyophthirius* was reported to trigger secretion of cortical mucocysts in vitro, and cause premature exit of parasites *in vivo*. While mechanisms underlying this phenomenon are largely unknown, parasite exit was suggested to be strictly dependent on i-antigen cross-linking rather than antibody [29]. Thus, carp epithelial chloride channel protein could be involved in the transport and distribution of the *I. multifiliis* GPI-anchored i-antigen protein in a similar way as TAP function, and in this way mediates host resistance to residency and re-infection by the parasite. However, structural and functional studies are required to elucidate the precise role of this protein in common carp-*I. multifiliis* interaction. In addition to their role in antigen presenting, the carp ABCC transporter may act as pattern recognition receptors to identify pathogen-associated molecular patterns of *I. multifiliis*, and therefore may be involved in carp resistance by mediating targeted export of toxins and inhibitors to infection sites. This could be in a way similar to multi-drug resistance mediated by drug-efflux pumps. In humans, overexpression of ABCC1 is attributed to multi-drug resistance [30]. Wheat gene Lr34 confers resistance to fungal pathogens. As Lr34 belongs to the pleiotropic drug resistance subfamily of ABC transporters, a possible mechanism of resistance may be the transport of toxic substances out of the plant cell [31]. A further known protein component of pathogen resistance is *Arabidopsis thaliana* PEN3, which is a putative ABC transporter. PEN3 mediates targeted export of toxins to penetration sites as a mechanism of disease resistance [31]. Hence, it is very interesting to investigate if such mechanisms contribute to fish resistance to parasites. This may explain why carp and other fish species are resistant against re-infection, and why some fish species are more resilient towards the parasite. For example, zebrafish are more resilient to *I. multifiliis* than rainbow trout and channel catfish [32]. The increased abundance of epithelial chloride channel protein is likely to mediate peptide recognition, antigen presentation and disease resistance.

The carp Ras GTPase-activating protein-binding protein 1 showed increased abundance (3.7-fold) at 9 dpe. Ras GTPase-activating protein-binding protein 1 is an element of the Ras signal transduction pathway

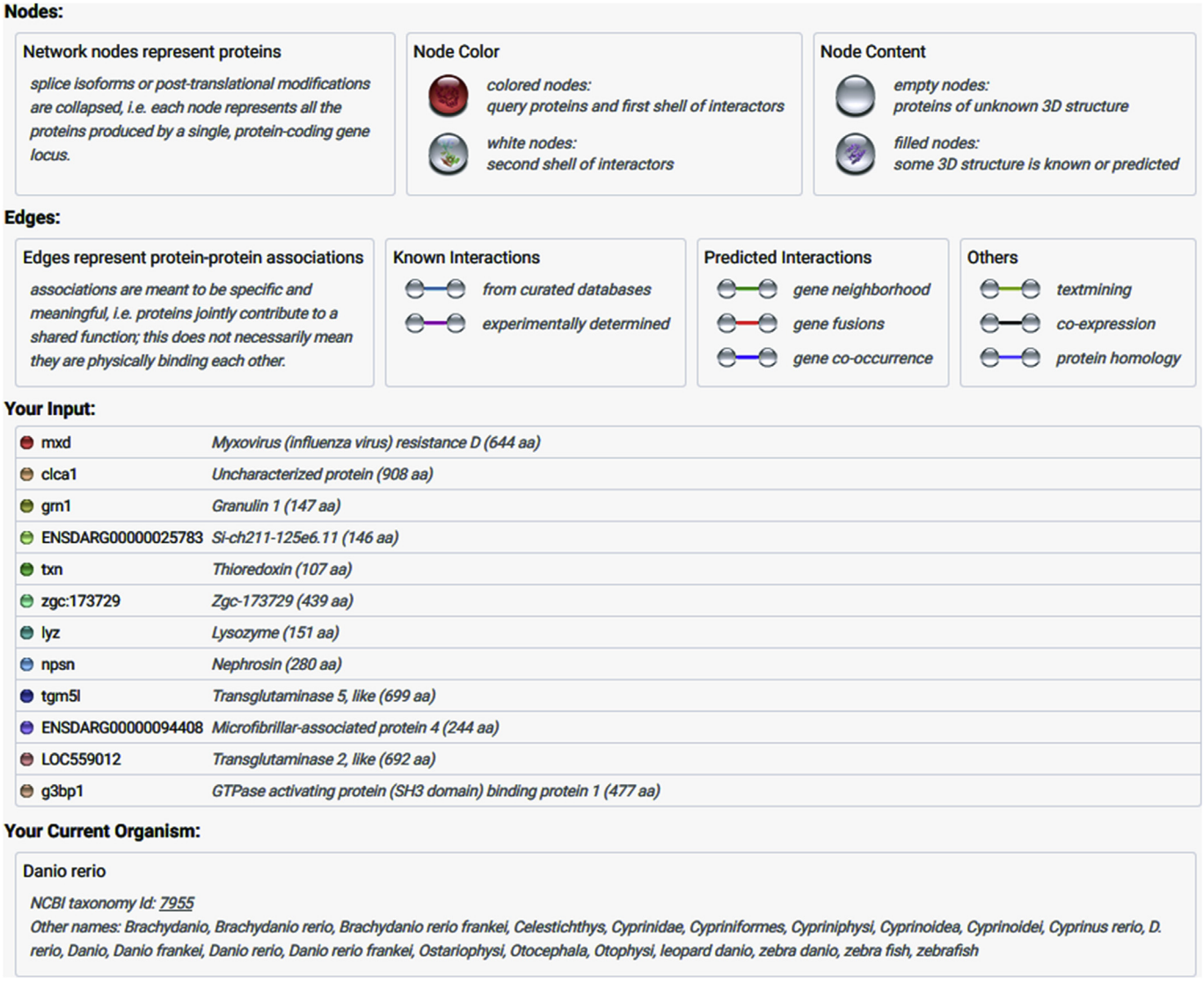

Fig. 3. The STRING screenshot shows the supplied set of signal transduction and immune-related proteins of common carp skin mucus. It shows details of protein abbreviation, node colour and edge interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

encoded by the G3BP1 gene. In catfish gills following columnaris infection, G3BP1 is down-regulated > 30-fold [33]. G3BP1, has been reported to be an important component in the assembly of stress granules during grass carp reovirus infection [34]. In another study, genes that encode Rho GTPases of the Ras-related proteins were dominant in Atlantic salmon during after piscine myocarditis virus infection [35].

The increased abundance of Ras GTPase-activating protein-binding protein 1 likely trigger signal transduction and activate the carp immune response against *I. multifiliis* as previously reported columnaris infected catfish [33].

Three interferon-induced GTP-binding Mx-like proteins were identified in carp skin mucus. The abundance of these proteins was induced significantly (4.8-fold) at 9 dpe.

Immune GTPases control vesicular traffic and protein complex assembly to stimulate oxidative, autophagic, membranolytic and inflammasome-related antimicrobial activities within the cytosol and on pathogen-containing vacuoles [36]. IFN-inducible Mx proteins are GTP binding proteins with intrinsic GTPase activity. The Mx proteins were originally identified as an inherited trait that confers resistance to influenza A. Mx proteins are now understood to be a group of broad-spectrum antiviral proteins that are effective against flu and influenza-like to gaviruses, bunyaviruses, rhabdoviruses, thogoto, coxsackie and hepatitis B viruses [36]. Their significance is best demonstrated by the ability of a human Mx1 transgene to completely rescue IFN type I (IFNα/β) receptor-deficient mice from influenza infection [36,37]. IFN-induced proteins were upregulated in Atlantic salmon during infection with piscine myocarditis virus [35]. Recently, interferon-induced GTP-binding protein Mx1 was detected in channel catfish and yellow catfish skin mucus [38]. Mx-like proteins were likely involved in binding and cross linking of the *I. multifiliis* FasR to FasL of carp neutophils and may explain the forced parasite exit. The increased abundance of carp interferon-induced GTP-binding Mx-like proteins likely supports oxidative, autophagic and inflammasome-related anti-parasitic activities to protect against *I. multifiliis*.

In this study, the carp high choriolytic enzyme 1 (nephrosin) showed increased abundance (6.8-fold) at 1 dpe. In a recent host-parasite interaction study, using an anti-*Tetracapsuloides bryosalmonae* monoclonal antibody linked to *N*-hydroxysuccinimide-activated spin columns, parasite and host proteins from the kidneys of infected and non-infected brown trout (*Salmo trutta*) were purified, which included high choriolytic enzyme 1, whose expression was suggested to be linked

with the infection of *T. bryosalmonae* [39]. Zebrafish nephrosin is specifically expressed in neutrophils and has been reported to endorse host resistance against bacterial infection [22]. Neutrophils play important roles in innate immunity and are mainly dependent on various enzyme-containing granules to kill invading microorganisms. Jørgensen [40] observed an average 3.4-fold increase in zebrafish neurophils, 24-h post-*I. multifiliis* infection, however, it then decreased, which was hypothesized to indicate that the cells were less attracted to the infection site and/or that the parasites evaded the immune system. Similarly, in the present study, the abundance of carp nephrosin was increased (6.8-fold) at 1 dpe then decreased (1.7-fold) by 9 dpe. In protein network analysis, nephrosin was associated with lysozyme C (Fig. 2). In accordance with previous observations [15], the lysozyme C-like protein abundance was also increased (7.9-fold) at 1 dpe. The increased abundance of both carp proteins (nephrosin and lysozome c) at 1 dpe was likely associated with carp neutrophils to promote protection against the pathogen, as a part of carp anti-parasitic defence. It has been reported that leucocytes were attracted to the sites of infection upon exposure to *I. multifiliis* [15,40,41]. However, this early, strong immune response was followed by a suppressive phase at 9 dpe, which was likely because of the increased anti-inflammatory response to protect against tissue destruction.

Three leukocyte elastase inhibitor (LEI)-like proteins showed increased abundance (6.9- to 10-fold) in infected carp skin mucus at 9 dpe. Within the parasite, a comparative genomic analysis revealed that the proteolytic repertoire of *I. multifiliis* consists of 254 protease homologs, which represent ~3% of the proteome [42]. However, host protease inhibitors modulate parasite protease activities and control a variety of critical protease-mediated processes, including the resistance to invasion by infectious agents. Carp infected with *I. multifiliis* produce elevated levels of A2M3, an isoform of A2M, a non-specific protease inhibitor of endogenous and exogenous proteases [43]. This strongly suggests that anti-proteases could be viable agents against pathogen infection. Differential transcription of the four isoforms of A2M was observed in the liver of carp infected with *I. multifiliis* [43].

Specifically, LEI belongs to the serpins family of proteins, and functions to limit and moderate protease activity, aimed at limiting host damage from inflammation and apoptosis, during the process of pathogen destruction by proteolytic activity [44].

LEIs have been identified in skin mucus of European sea bass (*Dicentrarchus labrax*) [44], and Atlantic cod (*Gadus morhua*) [45]. The increased production of LEI proteins has been suggested to be critical for neutrophil survival [46].

The abundance of all the LEI-like proteins in carp mucus was increased in a time dependent manner during *I. multifiliis* infection, which is likely essential for carp neutrophil survival.

Hence, the carp LEI proteins likely inhibit endogenous proteases to protect leucocytes against degradation and apoptosis activated by the apoptosis antigen (FasR) of the parasite, and protect against tissue destruction because of exogenous proteases secreted by the parasite throughout infection.

The abundance of five identified glutamine gamma-glutamyltransferase-like proteins was significantly increased (>40-fold). In zebrafish, genes correlated with immune responses, including glutamine gamma-glutamyl transferase, were induced due to *Mycobacterium marinum* [47]. Recently, two GSTs were identified in skin mucus proteome of European sea bass [44]. This protein was also reported in Atlantic cod skin mucus, and was differentially expressed after infection [45]. All 5 glutamine gamma-glutamyltransferase-like proteins showed increased abundance (3.5- to 8.7-fold) at 1 dpe and markedly augmented at 9 dpe (>40-fold) which suggests a protective antioxadative and anti-inflammatory role against tissue damage due to *I. multifiliis* invasion and development.

The thioredoxin-like protein showed significant increased abundance (4.2-fold) at 9 dpe in carp exposed to *I. multifiliis*. Thioredoxins are a part of antioxidant system present in cells and important for the control of redox potential, and were identified recently in the skin proteome of the European sea bass and gilthead sea bream [44,48]. The increased abundance of thioredoxin-like isoform X2 protein was likely aimed at reducing oxidative stresses and protecting carp against *I. multifiliis* infection.

Although several studies investigated fish-parasite interactions and fish immune responses against this parasite, they were mainly based on exploring cellular response and the modulation of gene expression of inflammatory cytokines of fish in response to *I. multifiliis*. The aim of the current study was to identify components of carp immune response which could have been missed by transcriptomic and gene expression studies and give insights into the post-transcriptional and post-translational regulation of carp skin mucus proteins. Indeed, this study successfully identified novel proteins that are highly likely involved in carp immune response to *I. multifiliis*. Nevertheless, the potential immune function of the identified carp skin mucus proteins is suggested after BLASTp analysis. Therefore, functional studies are required to explore their specific roles in common carp defense against deleterious effects of *I. multifiliis*.

5. Conclusion

Herein, entirely novel proteins in the context of the fish host response to *I. multifiliis* were identified such as nephrosin, epithelial chloride channel protein, Ras GTPase-activating protein-binding protein 1 and Leukocyte elastase inhibitors. The significant modulation of these 19 proteins was likely to coordinate and initiate a defence strategy against *I. multifiliis*. In addition, the presence of protein markers in fish skin mucus that have been previously predicted on the basis of modified mRNA expression was confirmed. These included lectin, lysozyme and granulin, which play a significant role in immune function. The identified lectins and LEIs with protease inhibitor activity are likely implicated in complement activation and regulation of proteolysis. The abundance of several anti-inflammatory proteins was increased likely to mediate an anti-inflammatory response to reduce tissue damage provoked by *I. multifiliis*. Taken together, the remarkable changes observed after 9 dpe with decreased abundance of the pathogen recognition and antimicrobial proteins (needed at initial stages of infection to promote defence against the parasite) lectin, nephrosin, lysozomes and granulin along with increased abundance of proteins with antioxidative and antinflammatory (needed for mediating protective responses) can be linked to carp immune modulation caused by the parasite (coincide with trophont maturation state at 1 dpe and 9 dpe and a switch from innate to adaptive immune responses). The results obtained using quantitative proteomics give not only knowledge on fish-*I. multifiliis* interactions, but also reveal biomarkers that are potentially useful for evaluation of host immune status and diagnosis of fish disease. Collectively, these results suggest that carp skin mucus plays important roles in activating immune response and protecting tissue from proteolysis and that the protein cargo of fish skin mucus contributes to its anti-inflammatory and protective properties.

Data availability

Shotgun proteomics data generated during the current study have been deposited in the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the PRIDE partner repository [49] with the dataset identifiers PXD011148.

Conflicts of interest

The authors declare that they have no competing interests. The authors declare that they don't have any commercial associations, current and within the past five years, that might pose a potential, perceived or real conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.078.

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