Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*)

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**A B S T R A C T**

Fish skin mucus serves as a first line of defense against pathogens and external stressors. In this study the proteomic profile of lumpsucker skin mucus was characterized using 2D gels coupled with tandem mass spectrometry. Mucosal proteins were identified by homology searches across the databases SwissProt, NCBI nr and vertebrate EST. The identified proteins were clustered into ten groups based on their gene ontology biological process in PANTHER (www.pantherdb.org). Calmodulin, cystatin-B, histone H2B, peroxiredoxin1, apolipoprotein A1, natterin-2, 14-3-3 protein, alfa enolase, pentraxin, warm temperature acclimation 65 kDa (WAP65kDa) and heat shock proteins were identified. Several of the proteins are known to be involved in immune and/or stress responses. Proteomic profile established in this study could be a benchmark for differential proteomics studies.

1. Introduction

*Cyclopterus lumpus* L., commonly known as lumpsucker/lumpfish, is a semi-pelagic fish distributed throughout the North Atlantic Ocean. This fish has been valued for its roe in fish food industry for decades [1]. Recently, use of this species as a delousing agent in salmon farms has gained interest. Lumpsucker is found to be a suitable candidate for delousing in waters even at lower temperatures where other cleaner fish might not thrive well [2]. Despite of the advantages of using lumpsucker as a cleaner fish there is a risk of transmission of diseases to the farmed salmon from infected lumpsuckers, needing further studies. Equally important is the understanding and management of the health and welfare of the lumpsucker itself. Bacterial infection is one major constraint in lumpsucker farming. There are several pathogens causing diseases in lumpsucker such as *Pasteurella* sp., atypical *Aeromonas salmonicida*, *Vibrio anguillarum*, *V. ordalii*, *Vibrio sp.*, *Tenacibaculum sp.*, *Paramoeba perurans*, *Gyrodactylus* sp. Infections were found to be more prevalent when fishes were stressed either by transport, vaccination and/or introduction to new environment [3]. Relatively little is known about lumpsucker’s biology and immune system, especially at the molecular level.

In fish, skin is one of the major sites for pathogen entry as it is a mucosal surface with living cells throughout. The skin mucus has a very important role in maintaining fish health, especially in intensive farming where level of stress and infections could be high. Skin mucus of fish contains a variety of immune relevant factors including lectins, lysozymes, calmodulin, immunoglobulins, complement, C-reactive proteins, proteolytic enzymes, anti-microbial peptides and proteins [4]. These factors form a biochemical barrier that serves as first line of defense against a wide range of pathogens. Characterization of skin mucus has been approached from different aspects focusing either on a particular protein of interest or a group of proteins. Recent studies use high throughput techniques for skin mucus characterization in fish. These include characterization of the i) proteome reference map of naïve Atlantic cod (*Gadus morhua*) skin mucus [5], ii) differential skin mucus proteome of Atlantic cod upon natural infection with *V. anguillarum* [6], iii) proteomic profile of discus fish (*Symphysodon aequifasciata*) skin mucus showing parental care [7], iv) proteomic profile of gilthead seabream (*Sparus aurata*) skin mucus [8,9], v) proteomics profile of European sea bass (*Dicentrarchus labrax*) [10], v) changes in protein composition of Atlantic salmon (*Salmo salar*) skin mucus followed by sea lice (*Lepeophtheirus salmonis*) infection [11], vi) skin mucus and sting venom of marine catfish (*Cathorops spixii*) revealing functional diversification of toxins [12].

Here we describe the skin mucus proteome of lumpsucker by using 2D gels coupled with mass spectrometry. We found immune relevant as well as stress physiology relevant proteins. These results could be useful for implementation of health and stress management strategies for production of a more robust fish.

2. Materials and methods

2.1. Fish and skin mucus sampling

Lumpsucker used in this study were provided by Arctic Cleanerfish, Stamsund, Norway. They were transported as newly hatched larvae,
further held at Merksvedbukta Research Station, Bodo, Norway, where they were start-fed with Gemma Micro and later fed with Amber Neptun of increasing sizes (1–4 mm). Both commercial feeds were from Skretting, Stavanger, Norway. The juveniles were raised on filtered seawater from 250 m depth, at 10–12 °C for the first 60 days and then the temperature was lowered to 7 °C until sampling. One-year-old fishes weighing approximately 700 g of varying length were anesthetized with MS-222 (70 mg/l) and killed by a blow to the head. For sampling of skin mucus the fish was kept on a plastic bag and massaged gently for a few seconds, discarding samples contaminated with feces. The mucus was transferred into tubes with the help of a spatula. The tubes were immediately frozen and stored at −80 °C until further analysis. All animal handling procedures were performed under the regulations set by National Animal Research Authority in Norway.

2.2. Sample preparation for 2-DE

Protein samples from skin mucus of eight fishes were extracted individually. For sample preparation the protocol of Wang et al. [13] was followed with few modifications. In brief, the skin mucus was thawed on ice and diluted with one volume of PBS containing 0.1% protease inhibitor (GE Healthcare, USA). The samples were sonicated (2×5 s) using an ultrasonic processor (SONICS Vibracell VCX750). Next, the sonicated skin mucus was centrifuged at 15,000g for 30 min, 4 °C to pellet the tissue debris and the supernatant was collected. A mixture of TCA (trichloroacetic acid), 10% w/v and 0.1% DTT (DL-Dithiothreitol, Sigma, USA) was added to the supernatant and incubated on ice for 30 min. The sample containing TCA and DTT was centrifuged at 10000g for 30 min, 4 °C. The pellet was resuspended in cold acetone containing 0.1% DTT and incubated at −20 °C for 45 min. The sample was centrifuged again at 10,000g for 30 min, 4 °C, the pellet obtained was air dried for 2–3 min and dissolved in rehydration buffer (9.8 M urea, 2% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), 20 mM DTT, 0.5% Bioylte (3–10), and 0.001% bromophenol blue, all from Sigma, except Bioylte from Bio-rad). The protein sample in rehydration buffer was used for two dimensional gel electrophoresis.

2.3. Two-dimensional gel electrophoresis

The protein content was estimated using Qubit® Protein Assay Kit and Qubit™ fluorometer (Life Technologies, USA) following the manufacturer’s protocol. 17 cm (pH 3–10), 10 mg/ml IPG strips (immobilized pH gradient, Bio-rad, USA) were rehydrated for 15 h using 80 µg of protein per strip. The rehydrated strips were subjected to isoelectric focusing in Bio-Rad Protean IEF cell to a total volt hours of 60,000 at a constant current of 20 mA/gel for 45 min. The sample was centrifuged at 10000g for 2 min and dissolved in rehydration buffer (9.8 M urea, 2% Bioylte, 2% CHAPS, 3–10, and 0.001% bromophenol blue, all from Sigma, except Bioylte from Bio-rad). The protein sample in rehydration buffer was used for two dimensional gel electrophoresis.

2.4. LC-MS/MS

A preparative gel was run with a protein content of 300 µg and stained with Sypro® Ruby as described by Kulkarni et al. [14]. The selected spots from the PDQuest analysis were excised manually on a blue light transilluminator (Safe Imager™ 2.0 Blue- Light Transilluminator, Life technologies, USA). The excised spots were trypsinized, reduced in gel, alkylated and subjected to LC-MS/MS analysis [15]. The analysis was performed with nanoAcquity ultra-performance liquid chromatography and Q-TOF Ultima global mass spectrometer (Micromass/Waters, MA, USA) at University Proteomics Platform, University of Tromsø, Norway.

2.5. Protein identification using bioinformatics tools

The LC-MS/MS analysis generated pl (powered keylogger) files by using the Protein Lynx Global server software (version 2.1, Micromass/ Waters, MA, USA). The plk files obtained were analyzed using MASCOT MS/MS Ions search (version 2.4.01) against SwissProt protein database (10 Jul 2015, 548872 sequences) and NCBI non-redundant database (10 Jul 2015, 6914658 sequences). In places where SwissProt or NCBI could not identify the protein, search was carried out against vertebrate EST database (10 Jul 2015, 54205008 sequences). The parameters set for protein identification were enzyme trypsin with one missed cleavage, fixed modification carbamidomethyl of cysteine and variable modification oxidation of methionine, peptide charge 2+ and 3+, peptide tolerance 100 ppm and MS/MS ion tolerance 0.1 Da. The search was performed for the taxonomic class, actinopterygii (ray finned fishes). All searches were carried out using the decoy search and the false discovery rate (FDR) were kept below 1% for both peptide matches above identity and homology threshold. Protein hits above significant threshold score and having at least one unique peptide sequence were identified.

2.6. Gene ontology (GO) enrichment analysis

For GO enrichment analysis UniProt IDs of identified proteins were retrieved from UniProt knowledgebase (UniProtKB). The UniProt IDs were submitted to PANTHER (www.pantherdb.org) to cluster the proteins into different groups relating to their biological process according to gene ontology annotation (GO terms). Only results with p < 0.05 were accepted. A protein-protein interaction network with a medium confidence score was created using string v9.05.

3. Results and discussion

At present, there are various techniques for mapping the proteome, however classical 2D gels still have their place in the field of protein and molecular biology. Benefits of using 2D gels include direct visualization of proteins giving a scope for assessment of the sample quality, ability to separate proteins even with small changes in pl and molecular weight, hence possibilities for identification of modifications in protein isoforms such as post translational changes or differences resulting from alternatively spliced mRNAs. It also serves as a powerful tool for identification of proteins from organisms with a non-sequenced genome by the help of de novo sequencing and homology searches [16].

In this study, proteins from naïve lumpsucker skin mucus were identified using 2D gels coupled with LC-MS/MS. Skin mucus proteins (100 µg/strip) from eight fishes were electro focused and ran on 12.5% polyacrylamide gels. A representative gel image is shown in Fig. 1. Out of 900 spots detected by PDQuest, only fifty highly expressed spots were excised for LC-MS/MS analysis but 40 spots were possible to identify using database searches. To our knowledge this is the first report on the skin mucus proteome of lumpsucker, C. lumpus. Lumpsucker’s genome has not been sequenced and very little information on the species is available in the databases. Thus, the proteins were
identified adapting homology searches restricting the BLAST searches
to the class Actinopterygii (ray finned fishes). Details of individual
proteins are listed in Table 1.

3.1. Immune and stress related proteins in skin mucus of lumpsucker

In this study spot 19 was identified as peroxiredoxin 1 (PRDX1). It
has also been reported in skin mucus of naïve gilthead seabream (S.
aurata) [8] and European seabass (Dicentrarchus labrax) [10]. Peroxiredoxins, also known as thiolredoxin peroxidase are cysteine-
based peroxidases grouped as 1-cys or 2-cys according to the number
of their cysteine-conserved residues [17]. These are antioxidant
proteins that protect the organism from toxic reactive oxygen species
(ROS) during oxidative stress (Fig. 2). It also participates in various
biological processes such as molecular chaperoning, hydrogen peroxide
mediated cell signaling and mitochondrial functions. PRDX1 is also
called natural killer enhancing factor A, has been implicated in immune
responses of many organisms. In fish the relatively high expression
level of PRDX1 in immune related tissues like spleen and kidney of
golden pompano (Trachinotus ocutus) suggests its role in immunity of
this species [18]. In infection studies, the expression of PRDX1 was
downregulated in Neoparamoeba perurans infected S. salar [19] and
Enteromyxum leei infected S. aurata [20]. Phagocytic cell produces
ROS to eliminate pathogens. Hence, downregulation of the PRDX1
gene may facilitate phagocytosis for removal of pathogens. Further, it
has been reported that extracellular peroxiredoxin 1 could act as
endogenous danger signal by binding to cell membrane sensors or
receptors [21].

Lectins are specific carbohydrate binding proteins involved in a
variety of biological roles. Here we identified two lectins namely
natterin (spot 22) and pentraxin (spot 46). Natterin was first isolated
from venom gland of Thalassophryne natteri [22] Natterin like
proteinaceous toxins (I and II) were purified from skin secretions of
oriental catfish (Plotosus lineatus) [23]. Natterin has a pore forming
toxin like domain with kinogenase activity [22]. The lectin like domain
in natterin is homologous to Jacalin domain identified in jack fruit.
Little work has been done on natterin in fish but jacalin, the plant
homologue, is reported to be involved in activation of human T-
lymphocytes [24] and apoptosis of B-lymphocytes [25] suggesting a
role in the immune system of fish. In mucus the lectin domain could
give direct interaction with pathogens and the pore forming toxin
domain could potentially result in lysis (Fig. 2).

Pentraxins, spot 46, are evolutionarily conserved proteins with a
variety of roles in host defense. As acute phase proteins, their role in
inflammatory responses and pathogen recognition make them impor-
tant markers of infection and inflammation (Fig. 2) [26]. Pentraxin is
found in skin mucus of common skate [27], surprisingly the skin gene
expression was not changed after in vivo challenge with E.coli.
This might suggest that the skin and mucus levels of pentraxin are constant,
or that this particular pathogen does not stimulate pentraxin produc-
tion in skin, but it does not exclude that proteins synthesis in liver
where human pentraxins are produced could change. Further studies
are needed to investigate mucus pentraxin function.

Spot 2 was identified as calmodulin. Previously calmodulin was
identified in skin mucus of sea lice infected Atlantic salmon [11] and
Vibrio anguillarum infected Atlantic cod [6]. This protein was also
purified from skin mucus of tilapia (Sarotherodon mossambicus), and
identified in mucus from European sea bass (Dicentrarchus labrax)
[10]. Calmodulin is calcium binding multifunctional protein highly
conserved in all eukaryotes. It is involved in cell signaling, stress and
immune responses. Calmodulin is an important calcium binding
protein found to be highly expressed in Antarctic notothenid fishes
when compared to warm water fish, this could indicate a protective role
against cold stress [28]. Further, studies reported that over expression

Table 1
MASCOT analysis details, gene symbols and physical parameters of identified protein spots from lump sucker skin mucus.

| Spot ID | Protein name (Species) | Gene symbol | Accession number | Observed P/MW | Apparent P/MW | St(%) | Protein score/Total score of Up(%) | SC(%) | Peptide sequence(%) |
|---------|------------------------|-------------|------------------|---------------|--------------|-------|------------------------------------|-------|---------------------|
| L2      | Calmodulin (Cyprinodon variegatus) | calm | Q6F78            | 15.3/7        | 16.0/4.09    | 29    | 49/75                              | 52    | KELGTVMRS            |
| L3      | Histrionicoteuthis hex划 (Didon) | kastk2b     | Q5B415           | 14.3/4.1      | 13.5/10.3    | 20    | 19/40                             | 5     | KDIEIVKVRK           |
| L4      | Predicted: Lipoxygenase-like (Xiphophorus maculatus) | lce1 | XP_005803374     | 17/9.5        | 21.3/1.94    | 48    | 9/57                             | 4     | KGDGVSEVLK           |
| L5      | Moxin, light polypeptide 9, like 1 (Salmo salar) | my9         | ACH170953        | 21.3/4.5      | 19.8/4.69    | 57    | 136/80                           | 29    | KEAFMMDIQNR          |
| L6      | Growth differentiatization factor 6-A (D. rerio) | gdf6a       | P85857           | 18.2/5.6      | 46.8/9.21    | 20    | 22/22                            | 3     | RFTDDEVDLFRE         |
| L7      | Glyceraldehyde 3-phosphate dehydrogenase (Caenorhabditis elegans) | gapdh      | P84677           | 10.8/5.4      | 42.6/4.95   | 19    | 51/51                           | 3     | KPLKEILASSPGASRR     |
| L8      | Nucleoside diphosphate kinase B (Mammus musculus) | nmp2e       | P85285           | 14.9/6.8      | 14.2/5.70   | 22    | 62/82                           | 9     | TPIAKIPGIVQGR         |
| L9      | Histone H1.2 (D. rerio) | histh1ne | Q4QRF4; A2V4D2 | 15.9/5.8      | 13.4/1.27    | 20    | 25/25                           | 5     | RELAIDQDFRT          |
| L10     | 60S ribosomal protein L11 (Lactobacillus plantarum) | rpl11       | Q9PYV7           | 21.6/5.8      | 20.6/10.07  | 33    | 37/37                           | 3     | KAEELFKE             |
| L11     | Glial fibrillary acidic protein (Caenorhabditis elegans) | gfaP       | P84677           | 11.9/5.1      | 42.6/4.93   | 24    | 92/64                           | 4     | RFLFEQKNXM           |
| L12     | Heme oxygenase 1 (Homo sapiens) | p455       | P84677           | 11.6/4.6      | 42.6/4.93   | 23    | 56/56                           | 4     | KLDLIAETYRK          |
| L13     | Peroxiredoxin 1 (Onychophora marina) | pred1       | AEA51065         | 26.1/6.4      | 22.9/6.2    | 57    | 266/129                        | 29    | RGLFVSRIDK           |
| L14     | Neurofilament H (Cricetulus griseus) | Ina1        | A1A555           | 28.4/4.4      | 26.6/6.74   | 59    | 70/70                           | 9     | KECAPVYNTNLEK         |
| L15     | Natriuretic peptide (Gastrovetus aurincola) | apo4         | CD995999        | 28.4/4.4      | 26.6/6.74   | 59    | 70/70                           | 9     | KECAPVYNTNLEK         |
| L16     | Tropomyosin isoform 1 (Thalassolaena nattereri) | tpm1       | Q90628; Q7T351_ | 29.9/7.8      | 278/45    | 23    | 115/29                          | 15    | KGAFEGIGSAPMKD        |
| L17     | Keratin, type I cytoskeletal 13 (Carcharhinus limbatus) | krt13     | Q8JP86           | 39.4/4.4      | 51/9.5     | 19    | 56/66                           | 1     | KLAADDFRT            |
| L18     | Predicted: F-actin-capping protein subunit beta isoforms 1 and 2 like isoform X (Oberoschmortus smithi) | capzb | XP_003441481     | 39.3/5.8      | 30.8/5.82   | 53    | 127/127                          | 26    | RSTLTNEIVYTGT        |
| L19     | 14-3-3 protein beta/alpha-A (D. rerio) | ywha5       | Q5PDR6; A3KND9   | 31.4/5        | 27.8/7.1    | 24    | 259/54                          | 18    | RVESSIQIQT            |
| L20     | 14-3-3 protein beta/alpha-1 (D. rerio) | ywha6       | Q6UF29           | 34.1/3.7      | 27.6/4.64   | 20    | 78/88                           | 7     | RENLSVAYKN           |
| L21     | Guanine nucleotide-binding protein subunit beta-2 like 1 (D. rerio) | gnb3b11 | O42248           | 36.7/8.2       | 35.5/6.0    | 30    | 93/93                           | 5     | KDVQLEFKQ            |
| L22     | Glial fibrillary acidic protein (Cricetulus griseus) | gfaP        | AC35053         | 45.7/6.8      | 36.3/6.20   | 43    | 87/87                           | 9     | KYYVESTGVFLSVEKA      |
| L23     | Charged multivesicular body protein 4C (D. rerio) | cmp4c       | Q6Q737; Q803U4   | 36.4/6        | 25/1.41      | 25    | 112/122                         | 9     | RETEEMLAAK           |
| L24     | 14-3-3 protein beta/alpha-B (D. rerio) | ywha8       | Q7T356           | 42.7/4.5      | 27.4/4.84   | 24    | 164/46                          | 16    | KRDSTLQMLR           |
| L25     | Keratin, type I cytoskeletal 13 (D. rerio) | krt13     | Q8JP86           | 68.1/7        | 51/9.31     | 24    | 31/31                           | 1     | KLAADDFRT            |
| L26     | Alpha-enolase (Thunnus obesus) | emo1        | B0H1J1; B0A0L7   | 57.8/6.1       | 47.5/7.2    | 26    | 639/586                         | 28    | RCNPFTVEVQLIRRK       |
| L27     | Nucleoside diphosphate kinase B (M. magellanicus) | nmp2e       | P85282          | 14.9/6.2      | 14.2/5.70   | 20    | 33/33                           | 9     | TPIAKIPGIVQGR         |

(continued on next page)
of the Antarctic notothenioid calmodulin gene in transgenic tobacco plants showed an increase in cold tolerance when grown at 4 °C for two weeks [29]. In Chinese mitten crab (Eriocheir sinensis) [30] and blue mussel [31] the calmodulin gene was significantly upregulated in gills and hepatopancreas under salinity and pH stress. This indicates that calmodulin might help to combat stress. Calmodulin could also have role in immune responses against pathogens. Silencing of calmodulin gene in Penaeus monodon made it susceptible to Vibrio harveyi infection resulting in increased mortality. This could be that silencing of calmodulin gene decreases the transcription of other immune related proteins required for the initiation of immune cascade [32].

Upregulation of this gene was reported in gills of Chinese mitten crab challenged with Edwardsiella tarda and V. anguillarum [30], and in hemocytes of Pacific white shrimp infected by V. parahemolyticus [33]. Thus calmodulin in lump sucker skin mucus might be involved in transduction of signals for downstream immune responses.

We also identified histone proteins, histone H2B (Spot 3) and histone H3 (Spot 13). Histones are major component of the nucleosomes and well known for their role in gene transcription regulation in eukaryotic cells. Studies have shown that there are also extranuclear histones present in mitochondria and on cell surfaces, with many physiologically important roles [34]. Histones released to the extracellular space serve as danger associated molecular patterns. Histones also serve as antimicrobial peptides that could either kill the pathogens directly or indirectly by blocking the DNA/RNA/protein synthesis (Fig. 2) [35,36]. H2B like protein isolated from skin mucus of Atlantic cod showed antimicrobial activity against E. coli [37]. Similarly H2B like protein in skin of Channel catfish (Ictalurus punctatus) showed antimicrobial activities against Aeromonas hydrophila and Seprolegnia spp. [38]. Further studies indicated that the level of histone like proteins were suppressed in channel catfish exposed to stress [39]. Histones are also identified in skin mucus of naïve European seabass [10].
Cystatin-B, also known as stefin-B, is a protease inhibitor, which regulates the activities of cysteine proteases. This protein is involved in both physiological and pathological conditions such as inflammatory responses (Fig. 2), protein homeostasis, antigen processing and metastasis. Spot 11 was identified as cystatin-B like protein. The presence of cystatin B in mucus might give protection against invading pathogen by inhibiting the cysteine proteases released from pathogens to promote their growth and proliferation. A protease inhibitor from pathogen by inhibiting the cysteine proteases released from pathogens in skin mucus might give protection against invading metastasis. Spot 11 was identified as cystatin-B like protein. The protein Cystatin-B was also involved in bacterial invasion of head kidney macrophages [42]. In naïve European sea bass [5], spot 60 and 61 were identified as a heat shock 70 kDa protein and heat shock cognate 71 kDa protein respectively. Heat shock proteins exists both intracellularly and extracellularly. Extracellular HSPs have been reported to act as immune modulators, that could be immunostimulatory or immunosuppressive depending on how they are encountered by the immune response network [51]. Heat shock protein 70 has been found in skin mucus of European sea bass [10] and gilthead seabream [8].

Enolases are a glycolytic enzyme, which also acts as plasminogen receptor, transcriptional regulator and cell associated stress protein (Fig. 2) [52]. Spot L34 was identified as alfa enolase in lumpsucker skin mucus. Alfa enolase serves as a stress marker in fish showing upregulation during hypoxic conditions in longjaw mudsucker (Gilllichthys mirebilis) [48]. Studies also showed upregulation of alfa enolase in Sparus aurata after in vivo LPS challenge [52].

The protein 14-3-3 was identified from four spots (26, 28, 32, 59) with varying pI and molecular weight suggesting several isoforms in skin mucus of naïve European sea bass [5] and Atlantic cod [10].

Warm temperature acclimation protein 65 kDa (WAP65) is homologous to mammalian hemopexin, a glycoprotein involved in transportation of heme from site of hemolysis. It could protect the skin against bacterial invasion by limiting available iron essential for bacterial proliferation and establishment. In this study spot 64 was identified as WAP65. Upregulation of WAP65 was observed in copper treated swordtail fish, Xiphophorus helleri [47] and upregulation of hemopexin like protein mRNA found in hypoxia induced longjaw mudsucker, Gillichthys mirebilis [48]. Differential expression of WAP65 was also observed due to warm temperature and bacterial infections in channel catfish [49]. Goldfish WAP65 contains a cytokine response element, suggesting a role in self-defense [50]. In naïve European sea bass WAP65 is present in skin mucus [10].

Heat shock proteins are highly conserved proteins involved in various stress responses including heat, heavy metal exposure, tissue damage, and pathogen infections (Fig. 2). These are molecular chaperones that helps the organism to repair the protein damage occurred due to adverse stress conditions. Spot 60 and 61 were identified as a heat shock 70 kDa protein and heat shock cognate 71 kDa protein respectively. Heat shock proteins exists both intracellularly and extracellularly. Extracellular HSPs have been reported to act as immune modulators, that could be immunostimulatory or immunosuppressive depending on how they are encountered by the immune response network [51]. Heat shock protein 70 has been found in skin mucus of European sea bass [10] and gilthead seabream [8].

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The protein 14-3-3 was identified from four spots (26, 28, 32, 59) with varying pI and molecular weight suggesting several isoforms in skin mucus of lumpsucker. These are highly conserved proteins found ubiquitously in animal tissues. They are signaling proteins associated
with osmoregulatory signal transduction in *Fundulus heteroclitus* gill epithelium [53]. This protein has also been involved in phagocytosis and microbial resistance in zebrafish. Knock down of this gene in zebrafish infected with *Staphylococcus aureus* showed decrease in survival rate than control fish indicates its role in bacterial resistance [54].

### 3.2. Other identified proteins

We also identified cytoskeletal proteins such as actin (spots 45, 57 and 58), Septin-2 (Spot 58), keratin (spots 33 and 24), F-actin capping protein (spot 25), myosin (spot 5). Actin is a dynamic protein that plays several roles in the cell. It is found to be involved in cell movement, phagocytosis (Fig. 2), cytokinesis and cytoplasmic streaming. Previously actin fragments have been identified in skin mucus of sea lice (*Lepeophtheirus salmonis*) infected Atlantic salmon (*Salmo salar*). Some of the proteins identified in lumpsucker skin mucus are enzymes involved in various metabolic pathways i.e. nucleoside diphosphate kinase B (spots 12 and 40), triosephosphate isomerase B (spot 23), glyceraldehyde 3-phosphate dehydrogenase (spot 30), malate dehydrogenase (spot 56) and ATP synthase (spot 42).

Identification of the proteins in skin mucus indicates a role in the extracellular space. Several delivery routes could be used to reach the outside of the cell [61]. That might be i) secreted through the ER-Golgi classical pathway, ii) released to the extracellular space by exosomes, iii) released by necrotic cells, iv) released from the endolysosomal pathway or v) by some unknown pathway yet to be discovered. Table 2 gives an overview of the identified proteins and of their previously known presence in extracellular space and/or skin mucus of fish.

### 3.3. Gene ontology analysis

The gene IDs for the 40 identified spots were obtained from UniProtKB for GO analysis. Gene IDs for all identified proteins could not be obtained for the fish model organism, zebrafish. Hence, the IDs used here were the human orthologs of the respective proteins identified in lumpsucker skin mucus except natterin-2, which do not have a human ortholog in UniProtKB. The GO biological process clustered the proteins into ten groups (Table 2) such as apoptotic process (GO:0006915), biological regulation (GO:0065007), cellular component organization or biogenesis (GO:0071840), cellular process (GO:0009987), developmental process (GO:0032502), immune system process (GO:0022376), localization (GO:0051179, GO:0001588), metabolic process (GO:0008152), multicellular organismal process (GO:0032501), response to stimulus (GO:0050896).

The protein-protein interaction network was created using String v9.05 employing the human UniProt IDs (Fig. 3). The interaction results need to be studied in an extracellular setting such as mucus,
establish if functional protein interaction network exist in mucus alone or in mucus interacting with skin cells and/or pathogens.

4. Conclusion

This study revealed the presence of several proteins that are involved in immune and stress responses in skin mucus of lumpsucker. Some of these proteins could be potential biomarkers for fish welfare. Thus, the proteome reference map of lumpsucker skin mucus could serve as a benchmark for future studies on lumpsucker, although this needs to be verified by additional research.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.12.016.

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