Characterization of skin immune and stress factors of lumpfish, *Cyclopterus lumpus*

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Preface

The thesis is submitted in fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the faculty of Biosciences and Aquaculture (FBA), Nord University. The different studies complied in this dissertation are original research performed at Nord University, Bodø over a period of three years. The studies were funded by The Norwegian Government, Nord University, and the projects 'Preventive healthcare of sea lice-eating lumpsucker—characterization of immune components in skin mucus' (VRI Nordland/NFR (2014-0476)), FHF – Rensvel (73577 FHF) and CycLus (Financed by Bjørøya AS).

The project team consisted of the following members:

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Martin H. Iversen, Associate Professor, FBA, Nord University: co- supervisor

Deepti M. Patel

Bodø, Jan 4th, 2018

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Paper I **Patel DM**, Brinchmann MF. (2017) Skin mucus proteins of Lumpsucker (*Cyclopterus lumpus*) skin mucus. Biophysics and Biochemistry reports

Paper II **Patel DM**, Bhide K, Bhide M, Iversen MH, Brinchmann MF Proteomic and structural differences in lumpsucker skin among dorsal, caudal and ventral region. Submitted

Paper III **Patel DM**, Iversen MH, Brinchmann MF. Effect of chronic stress on the skin proteome of lumpfish, *Cyclopterus lumpus*. Manuscript

Paper IV Hansen AB, **Patel DM**, Brinchmann MF, Iversen MH. The effect of chronic stress on primary, secondary and tertiary stress responses and its impact on animal welfare in lumpfish (*Cyclopterus lumpus*). Manuscript

Paper V **Patel DM**, Pinto N, Bizuayehu TT, Kristensen T, Iversen MH, Brinchmann MF. A multi-omics approach to assess the effect of temperature on lumpfish (*Cyclopterus lumpus*) skin. Manuscript

Abstract

Lumpfish, *Cyclopterus lumpus* is an important cleaner fish used for sea lice removal in Atlantic salmon cages. This biological method of sea lice removal is advantageous over chemical treatments that can influence the environment and lead to development of resistance in sea lice. Intensive fish farming induces stress on fish that affects health and welfare of the organism. Lumpfish is a relative new species in aquaculture, thus optimal farming condition and well-designed welfare program is yet to be established. In this dissertation I have investigated the skin/skin mucus of lumpfish under control and stressed conditions. Skin is one of the important mucosal tissues in fish that covers most of the body including fins. Fish skin acts as a first line of defence against a wide range of stressors and pathogens. Apart from immune defence skin also serves a multitude of function such as osmoregulation and mechanical protection.

Five studies were conducted, two on naïve lumpfish, two on lumpfish subjected to chronic stress and one temperature acclimation study, to investigate the response of skin/skin mucus. Proteome maps of skin and skin mucus of naive lumpfish, *Cyclopterus lumpus* were established using two-dimensional gels and mass spectrometry. The proteomic analysis showed many proteins that are involved in stress response, immune response, cytoskeletal organisation, energy metabolism, and localisation and developmental processes. Some important proteins identified in skin mucus are calmodulin, heat shock cognate 70 kDa, enolase alpha, natterin 2, apolipoprotein A1, pentraxin and histone proteins. These proteins are directly involved in or linked to immune and/or stress response pathways that might make them possible immune or stress markers.

Structural and proteomic differences were observed among dorsal, caudal and ventral regions in the skin of lumpfish. The structural differences were a thicker epidermis and higher goblet cell counts in the ventral region compared to the dorsal and caudal regions. Saccular like cells were observed in the dorsal and caudal regions, but did not

show any presence in the ventral region of the skin. Several proteins such as histone H4, heat shock cognate, parvalbumin, natterin-2, collagen alfa-1 and collagen alfa-2, 40S ribosomal protein and topoisomerase A and B were differentially expressed among the three regions of lumpfish skin.

Lumpfish were subjected to chronic stress by lowering the water level and refilling it back everyday for a period of 28 days. The results of this experiment indicated that the stressed group showed signs of allostatic overload type-2 due to oversensitivity to adrenocorticotropic hormone, and reduced negative feedback. This led to primary, secondary and tertiary changes in the stress response such as elevated plasma cortisol, reduced osmoregulatory ability and reduced growth. Two dimensional gel based proteomics were performed to find differential protein expressions in the stressed group compared to the control. The proteomic analysis showed a differential expression of calmodulin, heat shock protein 70kDa, histone H4, fatty acid binding protein, glutathione-S-transferase, 14-3-3 alpha/beta, keratin and guanine nucleotide binding protein. The heat shock protein showed reduced expression in the stressed group compared to control at all time points (7, 14, 21, 28 days) suggesting that due to the allostatic overload, fish were not able to maintain the balance and the homeostasis was hampered.

Two different temperatures 8 °C and 14 °C were used to study the effect of temperature acclimation on lumpfish skin. Differential protein and gene expressions were identified in lumpfish skin in temperature acclimation. The identified proteins/genes were involved in protein folding and degradation, protein biosynthesis, energy metabolism, cytoskeletal organization, cell proliferation and apoptosis. The results, of this study suggested that lumpfish could live at both the temperatures with few physiological adaptations.

In conclusion, the outcome of all the studies suggests that lumpfish skin/skin mucus have possible roles in immune and stress response as in other teleost species.

Abstract in Norwegian – Sammendrag på norsk

Rognkjeks, *Cyclopterus lumpus,* er en viktig rensefisk som benyttes for å fjerne lakselus fra atlantisk laks i oppdrettsmærer. Dette er en biologisk metode for å fjerne lakselus som har fordeler fremfor bruk av kjemiske behandlinger som kan påvirke miljøet rundt mærene og gjør lakselus resistente mot behandling. Intensivt fiskeoppdrett utsetter fisk for stress som påvirker dens helse og velferd. De optimale betingelsene for oppdrett av rognkjeks og bruk av den i laksemærer under gode velferd betingelser er fremdeles ikke etablert. I denne avhandlingen har jeg studert skinn/skinnmukus (slim) hos ubehandlede og stressede rognkjeks. Fiskens skinn er en viktig slimhinne som dekker det meste av fisken overflate inkludert finnene. Fiskeskinn er en del av førstelinjeforsvaret mot en rekke stressorer og patogener. I tillegg til å vær en del av immunforsvaret, har skinn også en rekke andre funksjoner slik som osmoregulering og mekanisk forsvar.

Fem studier ble utført, to på ubehandlede rognkjeks, to på rognkjeks utsatt for kronisk stress og ett temperaturakklimatiserings-studie for å undersøke responsen til skinn/skinnmukus. Proteomkart for skinn og skinnmukus fra ubehandlede rognkjeks *Cyclopterus lumpus* ble etablert ved bruk av todimensjonale geler og massespektrometri. Den proteomiske analysen identifiserte mange proteiner som er involvert i stressrespons, immunerespons, cytoskjelettorganisering, energimetabolisme og lokalisering- og utviklingsprosesser. Viktige proteiner identifisert i skinnmukus er blant andre kalmodulin, varmesjokk beslektet protein 70 kDa, enolase alpha, natterin 2, apolipoprotein A1, pentraxin and histon proteiner. Disse proteinene er direkte involvert i eller knyttet til immune- og/eller stressresponsveier som kan gjøre dem til mulige immune- eller stressmarkører.

Strukturelle og proteomiske forskjeller ble observert mellom de dorsal, kaudale og ventral områdene i skinn hos rognkjeks. De strukturelle forskjellene var en tykkere epidermis og høyere antall goblet celler i det ventrale området sammenlignet med de dorsale og kaudale områdene. Sakkulærlignende celler ble observert i de dorsale og

kaudale områdene, men var ikke tilstede i den ventrale delen av skinnet. Flere proteiner, slik som histon H4, varmestress beslektet protein, parvalbumin, natterin-2, kollagen alfa-1 and kollagen alfa-2, 40S ribosomal protein and topoisomerase A and B ble differensiellt uttrykt mellom de tre områdene i rognkjeksskinn.

Rognkjeks ble utsatt for kronisk stress, ved å senke vannivået og så la det strømme tilbake, hver dag i en periode på 28 dager. Resultatene av dette eksperimentet indikerte at den stressede gruppen viste tegn på allostatisk overbelastning type-2 på grunn av oversensitivitet ovenfor adenotropiske hormone og redusert negativ feedback. Dette medførte primære, sekundære og tertiaære endringer i stressresponsen som forhøyet plasmakortisol, redusert osmoregulatorisk evne og redusert vekst.. To dimensjonal gelbasert proteomikk ble utført for å finne forskjeller i proteinuttrykk i den stressede gruppen sammenlignet med kontroll gruppen. Proteomikkanalysen viste en forskjell i uttrykk av kalmodulin, varmesjokk-protein 70kDa, histon H4, fettsyrebindende protein, glutathione-S-transferase, 14-3-3 alpha/beta, keratin og guanin nukleotid bindende protein. Varmesjokkproteinet hadde redusert uttrykk i den stressede gruppen sammenlignet med kontrollgruppen ved alle tidspunktene (7, 14, 21, 28 dager). En mulig forklaring kan være at fisken ikke var i stand til å opprettholde metabolsk likevekt og homeostasen ble dermed hemmet på grunn av denallostatisk overbelastning.

To ulike temperaturer 8 °C og 14 °C ble brukt for å studere effekten av temperaturakklimatisering på rognkjeksskinn. Differensielt uttrykte proteiner og forandret genuttrykk ble observer i rognkjeksskinn i temperaturakklimiseringsforsøket. De identifiserte proteinene/genene er involvert i protein folding og- degradering, protein biosyntese, energimetabolisme, cytoskjelettorganisering, celle proliferering og apoptose. Resultatene av dette studiet indikerer at rognkjeks kan leve ved begge temperaturer med få fysiologiske tilpasninger.

For å konkludere, resultatet av alle studiene antyder at rognkjeksskinn/-skinnmukus har roller i immune- og stressrespons slik som i andre teleostarter.

1. Introduction

Skin is the largest organ in fish that is continuous throughout the body including fins. It protects the internal organs from the outer aqueous environment and maintains the body shape. It is a multifunctional tissue that serves an array of functions including sensory activities, protection from abrasion and mechanical injury, maintains the ionic balance by osmoregulation and protects against pathogens (Rakers et al. 2010). Epidermal mucus is an attributing feature in fish skin. The production and composition of mucus varies under various physiological conditions and/or during pathogen attack. The mucus is continuously replaced and it flushes away any adhering pathogen along with the water current preventing them from colonization. Fish skin and its mucus contain immune components that provide protection against various physical, chemical and biological stressors serving as primary line of defence (Esteban 2012). Study on fish skin is important for fish health and welfare and it has also applications in higher vertebrates as fish skin shares many features with mammalian gut (Xu et al. 2013).

1.1. Fish skin

1.1.1. Fish skin structure and components

Fish skin has two main anatomical layers, the outer epidermis and inner dermis. Skin of fish is non-keratinized and has a mucous layer that covers the epidermis. Keratinization is not common in fish as in terrestrial vertebrates, it is found only in few species that are capable of living outside water for limited periods such as lung fishes and in some species it is found in specific body parts that are prone to abrasion such as lips and adhesive organs (Tripathi and Mittal 2010, Alibardi and Joss 2003, Kumar Mittal and Whitear 1979).

The epidermal layer in fish consist of stratum superficiale, stratum spinosum and stratum basale. Stratum superficiale is the uppermost layer of skin epidermis in fish that contains simple squamous epithelial cells (Esteban and Rebeca 2015, Le Guellec 2004). These cells contain micro ridges that provide surface for mucus secretion and help to maintain the epidermal mucus layer. Orientation of these micro ridges is species specific (Fishelson 1984). Micro ridges provide a larger surface area and helps to retain the mucus substances secreted in skin surface and protects the fish from pathogens (Le Guellec 2004). The stratum spinosum is the intermediate layer of the epidermis. Most of the cells in stratum spinosum remain undifferentiated and helps in replacing the dead cells in the epidermis. Cells in stratum spinosum are capable to divide rapidly when necessary such as wound healing. The stratum basale is the innermost layer of epidermis that forms the basal lamina, which separates epidermis from dermis. Epidermis contains various cells such as malpighian cells, goblet cells, club cells, saccular/sacciform cells and sensory cells (Le Guellec 2004). The malpighian cells or filament containing cells constitutes a major component of epidermal cells. These cells can remove foreign materials from skin and initiate wound healing process. Cultured malpighian cells of Atlantic salmon (*Salmo salar*) could engulf bacteria (*Carnobacterium piscicola, Pseudomonas fluorescens and Aeromonas salmonicida salmonicida*) shows its phagocytic activity. Then the malpighian cells cover the wound rounding it up that leads to detachment of the wounded engulfed cells that subsequently sloughs away from the skin preventing the bacteria from colonization (Åsbakk 2001). Goblet cells produce mucus that contains proteins involved in immune response providing protection against pathogen and variety of stressors. The number of goblet cells varies depending on the physiological state of the fish. It is affected by stressful events such as changes in environmental parameters, interaction with toxins and pathogen attack (Esteban 2012). Club cells are identified in many fish species and have been found to have diverse role among different species. One of the study by (Pfeiffer 1977) mentioned club cells as alarm substances initiating a fright response in Ostariophysan fishes. In these fishes the by-products of club cells are exposed due to skin

penetrating pathogen, parasites or UV rays. Upon exposure it cues an alarming signal that brings the immune system in action (Chivers et al. 2007). Immunocytochemistry of skin of loaches (*Acanthophthalmus semicinctus* and *Botia horae*) and catfish (*Corydoras aeneus*) indicated the presence of chondroitin and keratin sulphates that could have healing properties for cell damage in fish (Ralphs and Benjamin 1992). The sacciform cells are found in epidermis of several teleost including Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod (*Gadus morhua*), brown trout (*Salmo trutta*) and in lumpfish epidermis (Ottesen and Olafsen 1997). These are round to oval with a single vacuole and almost empty cytoplasm. Sometimes the cytoplasm is filled with granular lumen to which the secreted products are released. These cells are bigger in size than usual goblet cells and do not respond to stains such as toluene blue, alcian blue, and periodic acid Schiff's stain. The cells appear either empty or have very weak eosinophilic reaction. Some fishes also possess glandular cells containing poison as in tetrodotoxin secreting glands in puffer fishes (Kodama et al. 1986). The thickness of epidermis varies according to size, sex, age, body site and from species to species. It is thicker in benthic species compared to pelagic species, this allows the benthic species to produce more protective substances and replenish dead cells fast (Glover et al. 2013).

The dermal layer consists of stratum spongiosum (laxum) and stratum compactum (Fig.1). Scales originate from dermal layer in case of fishes with scales. The stratum spongiosum contains fibroblasts, chromatophores, scales in fishes with scales, blood vessels and afferent nerves in a loosely arranged collagenous matrix (Rakers et al. 2010, Esteban and Rebeca 2015). The fibroblasts provide a structural framework and integrity to the skin. The chromatophores found in fish dermis are melanophores, irridophores and xanthophores. Melanophores protects the skin from harmful UV radiation, provides coloration and capable of changing the colour of skin (Sköld et al. 2008). Irridophores have roles in pattern organization in skin as in zebrafish (*Danio rerio*) (Patterson and Parichy 2013), irridophores in rainbow trout (*Onchorhynchus mykiss*) have ability to

reflect the light as it contains light reflecting platelets in the cytoplasm (Kelata 2009). Xanthophores are found to be involved in coloration of skin in zebrafish (Patterson and Parichy 2013). Lumpfish comes in many different colours. Larvae can be uniform brown, dark purple red, brown spotted or less common red (Moring 1994). Adult lumpfish has variable skin colour varying from green to purple in male and more green-yellow in females (Davenport and Thorsteinsson 1989) The inner layer of dermis, stratum compactum is composed of closely packed fibres of collagen as seen in species like turbot fish (*Psetta maxima*) (Faílde et al. 2014), and zebrafish (Le Guellec 2004). In scaleless fish the dermis has been found to be thicker than the fishes with scales. Thicker dermis in particular are the compactly arranged collagen fibrils in plywood like fashion in scaleless fishes such as catfishes provide strength against abrasion and protect the fibrils from splitting (Le Guellec 2004).

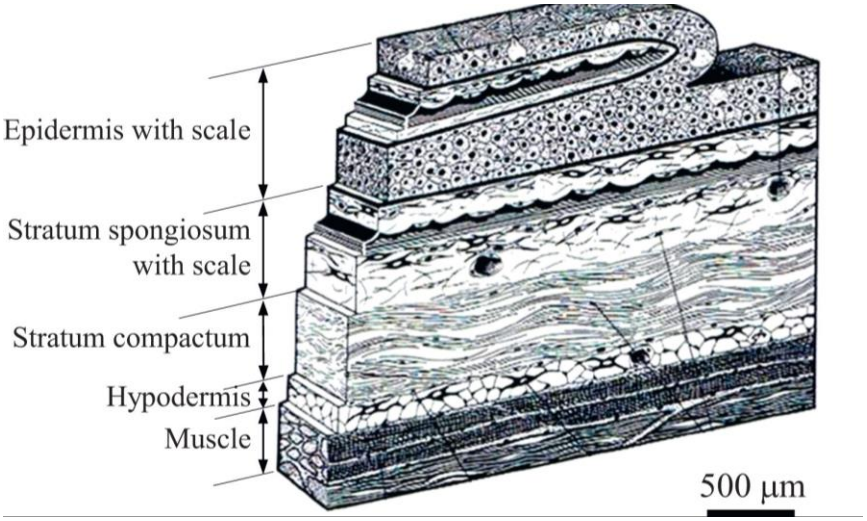

Figure 1. A generalized cross section of striped sea bass (*Morone saxatilis*) skin showing different layers as epidermis, dermis and hypodermis. Image is modified and reproduced from (Elliott 2000).

A hypodermal layer beneath the dermis is continuous with the muscle layer (Fig.1). This layer is more vascularized than the dermal layer and contains loosely oriented adipocytes, collagen and chromatophores. The adipocytes in this layer help the skin to be movable relative to its underlying muscle layer. The orientation of hypodermal layer varies

from species to species. In some species this layer is merged with the dermal layer making it a part of deep dermis rather than a separate layer as hypodermis (Rakers et al. 2010, Esteban 2012).

1.1.2. Innate immune components of fish skin

The immune system of an organism protects it from harmful pathogen attacks, stressors and maintains homeostasis. Fish is always in contact with its external milieu that is rich in microorganisms. It makes the fish more vulnerable to pathogen attack, which puts pressure on its epithelial barriers to develop defence mechanisms to fight against the invaders (Esteban 2012). The fish mucosal associated lymphoid tissue (MALT) is divided into four categories. Skin associated lymphoid tissue (SALT), gut associated lymphoid tissue (GALT), gill associated lymphoid tissue (GIALT) and nasal associated lymphoid tissue (NALT) (Xu et al. 2013, Tacchi et al. 2014, Salinas 2015). The MALT in fish has both innate and adaptive mechanisms. The innate and adaptive immune systems are not mutually exclusive, rather they work together to orchestrate the defence mechanisms. The innate immune system has a broad non-specific action in response to any stressors, whereas the adaptive mechanisms are more specific.

In fishes the innate immune system is well developed, that is able to fight against a variety of foreign invaders. The innate immune system is an early system, which is present before the adaptive immune system in development; it is early in evolutionary terms and also the first system non-self encounter. The innate immunity orchestrates the immune system and brings the adaptive systems into action to remove or kill the invaders and maintain the health status. Along with the epithelial barriers the innate immune components are divided into cellular and humoral components.

The fish skin contains mast cells/eosinophilic granular cells (ECGs), which expresses an array of functional proteins including antimicrobial peptides (AMPs) and plays a role in

inflammatory responses (Esteban 2012). In addition to this, macrophages and granulocytes, major players of innate immunity have also been identified in piscine skin (Gomez et al. 2013). A wide range of humoral components have been identified in fish skin and its mucus over the past few years. Those include enzymes, lectins, complement factors, antimicrobial peptides, transferrin, agglutinin, hemolysins, cytokines, chemokines and other proteins (Rajan et al. 2011, Patel and Brinchmann 2017, Cordero et al. 2015, Jurado et al. 2015, Esteban 2012). Many of the innate immune components could be used as potential biomarkers in health management and disease diagnosis in fish (Magnadóttir 2006).

Some of the enzymes identified in fish skin mucus are lysozyme, alkaline phosphatase, protease, cathepsin and esterase. Among all lysozyme is one of the most-studied enzymes in fish. Lysozyme has bactericidal activity that could kill both Gram positive and Gram-negative bacteria. It has the ability to opsonize the bacteria and trigger complement activation for subsequent phagocytosis and lysis of pathogenic bacteria. Lysozyme activity levels vary between species, sex, size, age, season and physiological conditions such as pH, salinity, temperature, and infections (Saurabh and Sahoo 2008).

Alkaline phosphatases have been identified in skin mucus of fish (Guardiola et al. 2014, Palaksha et al. 2008). Elevation of phosphatase enzymatic activity was found in skin mucus of sea lice infected Atlantic salmon (*Salmo salar*) (Fast et al. 2002). Increased phosphatase activity was reported during wound healing in catfish (*Heteropneustes fossilis*) skin (Rai and Mittal 1983). Alkaline phosphatases identified in human intestine are found to have roles in maintaining homeostasis of gut microbiota and have therapeutic potential against pathogens such as *Salmonella typhimuriu*m (Alam et al. 2014, Malo et al. 2010). As the skin mucosa of fish shares similar features with mammalian gut the alkaline phosphatases may have similar roles in skin of fish (Xu et al. 2013).

Proteases are protein-hydrolyzing enzymes that are categorized as serine, cysteine, aspartic and metalloproteases. Serine proteases are involved in the complement activation pathway. Trypsin (serine protease) and cathepsin B and L (cysteine proteases) released upon bacterial infection could kill the bacteria or could be able to modify the property of the mucus and slough away the extra mucus with the infectious agent (Aranishi et al. 1998). High level of enzyme activity (lysozyme, alkaline phosphatase, peroxidase, proteases, esterases) and bactericidal activity against tested pathogens including *Photobacterium damselae, Vibrio angillarum, V. harveyi* have been observed in gilt head sea bream (*Sparus aurata*) (Guardiola et al. 2014).

Lectins are specific carbohydrate binding proteins that play a vital role in immune defense. These proteins are involved in pathogen recognition, opsonization and phagocytosis, bacterial agglutination and complement activation. Galectins isolated from teleost fish, congerin in conger eel (*Conger myriaster*) and galectin-1 in Atlantic cod (*Gadus morhua*) could recognize and agglutinate pathogenic *Vibrio anguillarum* and some other marine bacteria (Kamiya et al. 1988, Rajan et al. 2013a). Mannose binding natterin like protein with kinogenase and pore forming activities was isolated from skin mucus of Atlantic cod that could hemagglutinate as well as agglutinate bacteria (Rajan et al. 2017a). The lectin calnexin from channel catfish (*Ictalurus punctatus*) was found to be associated with class II major histocompatibility complex (MHC). This indicates the vital roles of lectins in fish immunity.

Numerous antimicrobial proteins/peptides (AMP) have been discovered in a variety of tissues from different groups of organisms. Also, the list of AMPs identified in fish skin mucus is increasing year by year. AMPs are small peptides that could kill the microorganism directly by pore forming activity or could inhibit their growth e.g. by blocking the protein synthesis. AMPs are crucial to maintain the immune homeostasis by eliminating the pathogenic microorganisms while conserving the commensals. Skin mucus

antimicrobial peptides onchorynchin II and III, histone 2A, pleurocidin, hepcidins, defensin, cathelicidins, apolipoproteins could show antimicrobial activities against a variety of pathogens including fungus, Gram positive and Gram negative bacteria (Rakers et al. 2013).

Complement proteins are responsible for range of immune functions including activation of the adaptive response, promotion of inflammation, elimination of pathogens, apoptotic and necrotic cells (Gomez et al. 2013). Complement C7 expression has been found in grass carp skin (Shen et al. 2012), C3 has been identified in skin mucus of gilthead sea bream (Cordero et al. 2016).

The pathogen associated molecular patterns (PAMPs) have important role in pathogen recognition and initiation of immune response. Toll like receptors (TLRs) are well-recognized cell surface receptors in the innate immune system. Upon pathogen infection these receptors recognize the foreign molecular patterns of invaders and initiates signal transduction that triggers Myd88 resulting in release of cytokines (Rebl et al. 2010). Several TLR's expression were regulated in skin of catfish (*Ictalurus punctatus*) upon *Ichthyopthirius multifilis* infection suggesting their role in immune functions (Zhao et al. 2013).

1.2. Stress factors associated with fish farming

1.2.1. The stress response in fish

Stress could be defined as a situation when the organism's dynamic equilibrium known as homeostasis is disturbed by the influence of stressors (Chrousos 1998, Selye 1973). Stressors in fish farming could be chemical (exposure to pollutants and toxins, changes in environmental parameters like temperature, salinity, and oxygen), physical

(handling, capture, confinement, transport), biological (infection and diseases) and perceived stressors (presence of predators).

The physiological responses of fish to stressors are categorized as primary, secondary and tertiary responses. The primary response involves the initial signals send by the neuroendocrine system upon encounter of any kind of stressor. This leads to release of catecholamines from the chromaffin tissues and stimulation of the hypothalamic-pituitary-interrenal (HPI) axis to release corticosteroids into the blood stream. The secondary response involves metabolic and cellular changes such as change in glucose or lactate levels, increased production of stress proteins like heat shock proteins. It also involves changes in haematological parameters, disturbance in osmoregulation, and immune responses against stressors such as antibody production or lysozyme activity. The tertiary response involves changes in performance of the animal such as growth rate, swimming activities, disease resistance, change in feeding behaviour and aggression (Barton 2002, Wendelaar Bonga 1997, Wendelaar Bonga 2011). However, beyond this simplified version of stress response, the response varies depending on the magnitude and duration of exposure to a stressor. The response may affect all levels including molecular to biochemical and population to community.

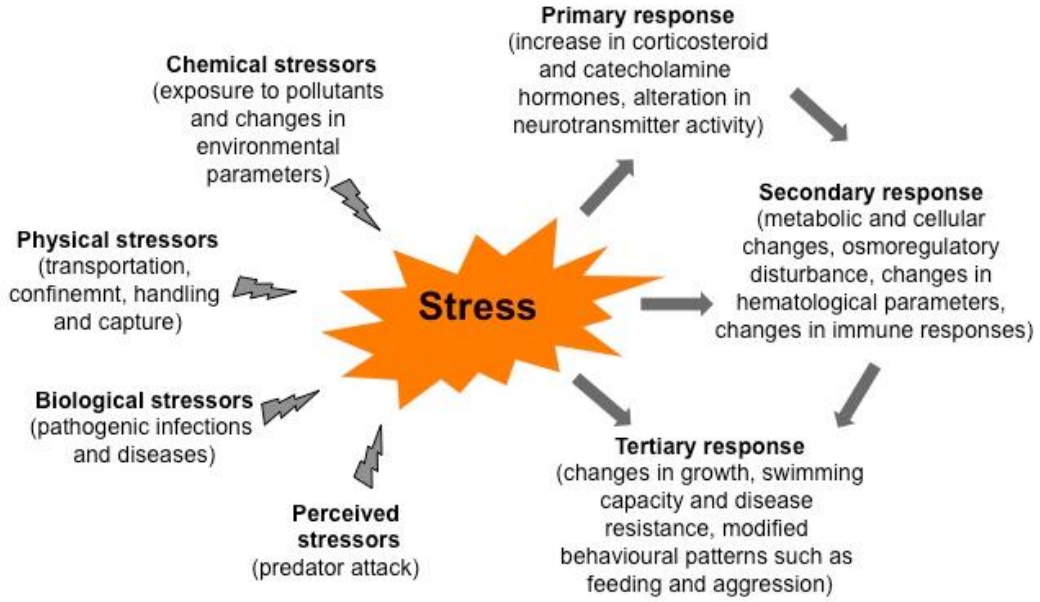

Figure 2. Stress responses on fish (primary, secondary, and tertiary) evoked by stressors (physical, chemical, biological and perceived) to maintain homeostasis. The figure is modified and redrawn from (Barton 2002).

Stress is not always detrimental; it is a non-specific response of the fish to re-establish the disturbed equilibrium caused by stressors, to maintain homeostasis (Selye 1973, Barton 2002, Chrousos 1998). If the stress is severe and persists for longer period, the fish might not be able regain the homeostasis. In such case the stress responses become maladaptive and have adverse effects on fish health and welfare (Barton 2002).

A stress event initiates immune response in an organism that includes synthesis of proteins such as complements, initiation of phagocytosis, production of antibodies, and expression of proteins involved in immune responses. In long-term stress, providing the resources to accomplish all these mechanisms to repair the damage for a prolonged period becomes exhaustive for the organism. After certain time the organism lacks the ability to meet the demand of the system to carry out the defence against the stressor (Tort 2011). On the other hand, in short-term stress all the immune mechanisms might not be affected except some essential mechanisms such as phagocytosis. Also, short-term

stress could have stimulative effect on immune system that leaves a memory of the stress episode that help to fight against the similar encounters in future (Tort 2011, Dhabhar 2009, Dhabhar and McEwen 1999).

1.2.1.1. Allostasis

Allostasis is an alternative concept that goes beyond the general concept of homeostasis. The concept of allostasis is to achieve stability through change (McEwen and Wingfield 2003). According to McEwen (2005) allostasis clarifies the uncertainty associated with the term "stress". Thus, it is summarized that homeostasis is essential for life whereas allostasis is a process that supports homeostasis and is essential for maintenance of balance when there is change in the environment or during changing of life history stages (McEwen 2005). Allostatic state refers to the adaptive response to stress and increase in concentrations of primary mediators such as corticosteroids, catecholamines and cytokines, in response to any stressor can be found. But when the exposure to stressor continues for a longer period, and the body is forced to adapt to the adverse physiological situations, it is called allostatic load (McEwen 2000). When the stress overpasses the tolerance limit and the organism cannot cope well with the changes, the allostatic load becomes allostatic overload. The allostatic overload can be divided into two categories; i) overload type 1, which could be adaptive if the organism overcomes the stress and balance is regained ii) overload type 2 which could be detrimental for the animal if the level of stress goes beyond the tolerance limit (Wingfield 2005, McEwen 2005, Goymann and Wingfield 2004, Ramsay and Woods 2014).

1.2.2. Studies on the effect of stress on fish

The stress and/or immune responses of fishes have been studied by exposing them to stressors such as crowding, exposure to heavy metal and toxicants, transportation, manipulation of the environmental parameters such as oxygen, ammonia levels, salinity and by infection studies against pathogens. Methods to assess stress in fish is by

measuring cortisol levels in serum or skin mucus, measuring the gene/protein expression of stress and immune related factors (Belanger et al. 2001, Guardiola et al. 2016, Vatsos et al. 2010, Sun et al. 2017, Liu et al. 2017, Gandar et al. 2017a, Iversen and Eliassen 2014).

To cope with the stressful condition and re-establish homeostasis the stress response can initiate the immune response by supressing or activating certain pathways, that involves changes in gene and protein expression levels. Identification of the genes/proteins expressions, which are regulated by any stressful event, could be achieved by comparing the proteome or transcriptome of stressed individuals with non-stressed ones. To estimate the level of known immune and stress related genes in tissues of stressed fish is a widely used method in aquaculture research. Juvenile turbot (*Scophthalmus maximus*) exposed to increased ammonia levels and crowding stress showed increased plasma sodium (Na^+) levels and high expressions of heat shock protein 70 (HSP 70), HSP 90, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) genes in blood, gill and liver samples compared to control fish (Liu et al. 2017). High stocking density stress in large yellow croaker (*Larmichthyes crocea*) initiated the complement, and coagulation cascades followed by chemokine and toll like receptor signaling pathways suggesting the immune responses due to induced stress. These immune responses could provide disease resistance against bacterial pathogens at early stage. However, prolonged exposure to stress weakens the immunity in fish (Sun et al. 2017). Chronic stress slows down the wound healing capacity in fish as observed in gilthead sea bream (*Sparus aurata*) (Mateus et al. 2017), and negatively affects the antibody production in Atlantic salmon (*Salmo salar*) (Iversen and Eliassen, 2014).

Increased plasma cortisol levels, reduced catalase and super oxide dismutase activities and reduced metabolic levels were observed in gold fish (*Carrasius auratus*) exposed to a combined pesticide and temperature stress (Gandar et al. 2017a). The liver

proteome of gold fish exposed to combined pesticide and temperature stress showed differential expression of proteins such as heat shock cognate 70, heat shock protein 60, sorbitol dehydrogenase, fructose biphosphate aldolase, tubulin, and inter filament protein (Gandar et al. 2017b). Water reduction, transportation and handling stress in farmed white sturgeon (*Acipenser transmontanus*) significantly elevated the plasma cortisol levels compared to the resting sturgeons indicating stress as a result of the imposed stressors (Belanger et al. 2001). Measurement of skin mucus cortisol levels could be a non-invasive method to assess stress response in farmed fishes. Cortisol levels in the surrounding water and skin mucus of gilthead sea bream (*Sparus aurata*) were significantly correlated to the serum cortisol levels when the fishes were exposed to acute crowding, anaesthetic agents and air exposure. Immunoglobulin M (IgM) and immune related enzymes peroxidases and proteases were identified in skin mucus and serum of stressed fishes suggesting their roles as stress indicators (Guardiola et al. 2016). Stressors can impact the mucus production in skin and sea bass exposed to high nitrate levels and low oxygen levels showed significant increase in number of skin mucus cells compared to the control group (Vatsos et al. 2010).

1.2.3. Temperature and its effect on skin and skin mucus

Temperature has a significant effect on the welfare of ectothermic (exception; tuna, genus- *Thunnus*) animals like fish (Altringham and Block 1997). Fishes have their optimum ambient temperature for growth and survival; however, they can also survive in a wider range of temperature. Lumpfish can survive in temperatures from 4 °C till 16 °C (Nytrø et al. 2014). The results from Nytrø et al (2014) showed that the optimum temperature for growth decreases with increasing size of lumpfish. Fishes in the wild can regulate temperature by moving closer to shore or to the surface to increase temperature and offshore or to deeper waters to reduce temperature (Freitas et al. 2015). In aquaculture pens, the movement is restricted even if, depending on the depth of the pen, the fish can still move vertically.

In aquaculture, temperature plays an important role in relation to the pathogenic infections. Some pathogens are temperature modulated and infections start at low or high temperatures such as winter ulcer, a bacterial infection that develops skin lesion (Karlsen et al. 2017), cold-water strawberry disease or red mark syndrome in rainbow trout (Verner-Jeffreys et al. 2008) that are all seen in fishes when the water temperature decreases. Columnaris disease occurs in warmer temperatures and causes lesions, which destroys the skin, gill and muscle tissues (Wakabayashi 1991).

Temperature affects the mucous cell counts in fish skin. A study on channel catfish subjected to an acute temperature drop (22 °C to 10 °C) showed subsequent decrease in mucuos cells in the skin on day 3 but the mucous cell count were back to normal after 6 days, showing its acclimation to low temperature. However, when the channel catfish was subjected to both, acute temperature drop and *Saprolegnia*, the mucous cell count did not go back to normal and the skin appeared dry (Quiniou et al. 1998).

A change in temperature affects the chemical composition of skin in fish. Atlantic salmon raised at 4 °C, 10 °C and 16 °C showed variation in its protein, fatty acid and moisture content. Fishes kept at 16 °C showed high protein and fat content compared to fishes at 4 °C and 10 °C (Jensen et al. 2015a). Jensen et al (2015) also suggested that the epidermal thickness of skin was higher at 4 °C compared to that found at 10 °C and 16 °C. Temperature also plays a role in wound healing, Atlantic salmon kept at 12 °C showed faster wound healing than fishes kept at 4 °C (Jensen et al. 2015b). Studies have also suggested that low temperatures suppress the immune system of fish (Bly and Clem 1992, Le Morvan et al. 1998). Significant changes in several immune factors such as lysozyme, transferrin, alkaline/acidic phosphatases, immunoglobulin M, interleukin 1-beta, hepcidin and superoxide dismutase (SOD) was observed in turbot (*Scopthalmus maximus*) skin mucus subjected to high water temperature, suggesting regulation of the immune system in turbot skin to cope with high temperatures (Huang et al. 2011).

1.3. Lumpfish

1.3.1. Geographical distribution and biology

Lumpfish, *Cyclopterus lumpus* Linnaeus, 1758 also known as lumpsucker is a scaleless scorpaeniform fish distributed throughout the North Atlantic Ocean. To the west of Atlantic Ocean, it is found in waters of Greenland, Hudson Bay, Newfoundland, the Gulf of Saint Lawrence, New Brunswick, and Nova Scotia. Towards the European coast it is common in Iceland, Jan Mayen, Spitzbergen, in Barents Sea and White sea along the coasts of Norway, Sweden, Denmark, Belgium, France, Portugal and Spain (Vasconcelos et al. 2004, Bañón et al. 2008, Davenport 1985). Lumpfish has a compressed body with a large humped dorsal crest that overgrows the first dorsal fin. The pectoral fin is modified into a sucking disc that allows it to anchor itself to rocky surfaces, vegetation, algae or any other objects in its surroundings (Davenport 1985, Mecklenburg and Sheiko 2003). Lumpfish are semi pelagic fish that moves from offshore to coastal water for spawning during spring and early summer. The female lays demersal eggs in batches and male guards the eggs until hatching (Kennedy 2015b, Mitamura et al. 2012). The eggs are hatched near the shore and the juveniles stay there for a year before migrating to offshore pelagic and mid waters. Lumpfish are sexually dimorphic in their body sizes female being larger in size compared to males (Davenport 1985). Lumpfish feed on benthic organisms such as polychaetes, crustaceans, molluscs and some of the slow moving pelagic organisms such as medusa and ctenophores (Mecklenburg and Sheiko 2003).

Figure 3. Juvenile lumpfish (*Cyclopterus lumpus*). Photo Dr. Martin H. Iversen, Nord University

1.3.2. Importance of lumpfish

1.3.2.1. Lumpfish as biological control of sea lice (*Lepeophteirus salmonis,* Krøyer)

Atlantic salmon (*Salmo salar*) is a highly prized and economically important species in Norwegian aquaculture. Infestation of the ectoparasite *Lepeopthrius salmonis* in Atlantic salmon remains as one of the major health and welfare issue. The treatment of sea lice demands a considerable amount of money, approximately €430 million in 2015 in Norway, excluding loss of productivity (Iversen et al. 2015). The use of cleaner fish has evolved as a green alternative compared to the use of antiparasitic chemo theraputants that develops resistance in sea lice and imposes harmful environmental effects (Powell et al. 2017). The use of this biological method could be cost effective and less stressful on salmon compared to use of chemicals (Liu and Bjelland 2014, Treasurer 2002). Wrasse has been used for sea lice control way before lumpfish was introduced as cleaner fish. In particular ballan wrasse has been shown to be an effective delousing agent in terms of efficacy, sustainability and fish welfare (Skiftesvik et al. 2013). However, wrasse becomes inactive at low temperatures showing loss in appetite and reduced metabolism at

temperature below 6 °C (Treasurer 2002, Sayer and Reader 1996, Sayer and Davenport 1996). Lumpfish can thrive well at low temperature such as 4 °C and can be used in salmon farms in as little as 4 months old compared to ballan wrasse that takes approximately 1.5 years to be useful for delousing (Powell et al. 2017, Nytrø et al. 2014, Helland et al. 2014). Lumpfish' efficacy of grazing sea lice in salmon cages was studied by Imsland et al. (2014). Juvenile lumpfish of approximate weight 54 g were stocked at 10 % and 15 % of density of Atlantic salmon in cages over a period of 54 days. The efficacy was evaluated by estimating gastric lavage of lumpfish. The results revealed that 28 % of lumpfish had ingested sea lice on the last sampling day: lumpfish gave 93-97 % reduction in adult female sea lice compared to the control cages without lumpfish (Imsland et al. 2014a). Lumpfish are opportunistic feeders that feed on sea lice as well as on salmon pellets and organisms attached to the cages (Imsland et al. 2015b, Powell et al. 2017).

Success of multispecies aquaculture highly depends on compatibility of the species with each other whether all species are raised as food fish or using cleaner fish for removal of sea lice in Atlantic salmon cages. The behavior of lumpfish towards Atlantic salmon in cages was studied by using underwater cameras in the cages (Imsland et al. 2014b). Although the lumpfish in cages did not spend most of the time on grazing sea lice, still a significant amount of sea lice were removed in experimental cages. The important behavior with concern to welfare of both lumpfish and salmon was that they could co exist in the same cage without showing any antagonistic behavior towards each other (Imsland et al. 2014b, Imsland et al. 2015a).

1.3.2.2. Lumpfish as seafood

Lumpfish roe has been a delicacy since decades. Along the Norwegian coast lumpfish has been commercially fished for its roe way before it was introduced as a biological method of delousing. The females are targeted for capture during the spawning season when they migrate from offshores to coastal zones during spring season

(Mitamura et al. 2012). Greenland and Iceland have the highest contribution to lumpfish roe followed by Canada and Norway. The roe constitutes almost 25 % of the weight of the female fish (Kennedy 2015b). The lumpfish caviar is a low cost alternative to highly prized true sturgeon caviar that is receding due to overfishing of sturgeons.

1.3.3. Challenges in lumpfish aquaculture

The use of lumpfish in salmon farms is steadily increasing. Farming of lumpfish has started in Norway in recent years. In 2016 the "The Norwegian Directorate of Fisheries" issued 65 licenses to companies for lumpfish production along the coast of Norway. Total number of lumpfish used in Atlantic salmon cages in 2016 was 15,784,000 of a value of 316,162,000 NOK (www.fiskeridir.no). All lumpfish used for sea lice removal in salmon cages are farmed not wild caught. However, due to non-availability of a suitable breeding program, the brood stocks are mostly wild caught (Hjeltnes B and Walde 2017).

Stress and diseases are the top challenges in any intensive aquaculture, so as in lumpfish farming. Lumpfish farming is in its initial phase of development to establish suitable health management practices to avoid stress and infection, to develop suitable feed and feeding strategies for optimal growth, to develop breeding and spawning program in captivity, and to develop effective vaccines against commonly occurring diseases (Powell et al. 2017). A high stocking density in intensive farming creates physiological stress that weakens the immune system of the organism. Stress during capture, storage, transportation from hatchery to sea, and handling are some of the reasons of mortality in lumpfish (Powell et al. 2017). According to Norwegian Fish health report 2016 lumpfish mortality increased dramatically post handling and treatment against pathogens (Hjeltnes B and Walde 2017).

Disease is one of the main problems in lumpfish farming. Bacterial pathogens such as *V. anguillarum, V. ordalii,* atypical *Aeromonas salmonicida*, *Pasteurella sp*. and

Tenacibaculum spp. are identified in lumpfish (Alarcón et al. 2016a, Småge et al. 2016, Marcos-López et al. 2013, Hjeltnes B and Walde 2017). Frununculosis caused by *Aeromonas salmonicida* subsp. *salmonicida* was reported in both farmed and caged lumpfish kept with salmon. However, salmon being vaccinated against furunculosis did not show any signs of diseases. Viral pathogens IPN and VHS was detected in farmed lumpfish in Norway and Iceland (Powell et al. 2017). *Paramoeba perurans* the causative agent of amoebic gill disease has been isolated from lumpfish that can transfer the disease to Atlantic salmon (Haugland et al. 2017). Lumpfish has also found to be infected with *Ichthyobodo spp.*, *Gyrodactylus sp*., , flavivirus, *Caligus elongatus* and fungal pathogens *Nucleospora cyclopteri, Exophiala sp*. (Mitamura et al. 2012, Øines and Heuch 2007, Alarcón et al. 2016b). Compared to 2015, the mortality has increased due to bacterial pathogens and flavivirus infections in lumpfish (Hjeltnes B and Walde 2017).

Being together with Atlantic salmon in the sea cages there is always a risk of disease transmission from lumpfish to the salmon (Haugland et al. 2017). Thus, the attention towards health and welfare issues of lumpfish has increased in recent years to produce robust individuals. Even if the welfare and routine health management strategies are more or less similar for most of the aquaculture species, still every individual species differ in their biological needs. Therefore, it is important to have basic knowledge about the biology and immune system of individual species in order to develop robust health strategies. Research on the immune system and vaccine development for lumpfish has become a top research priority. Presence of specific antibodies was detected in immunized lumpfish against bacterial pathogens isolated from disease outbreak in lumpfish (*V. anguillarum* and atypical *A. salmonicida, Pasteurella* like) suggesting that vaccine development could be possible against specific pathogens (Rønneseth et al. 2015). Significant proportions of farmed lumpfish are vaccinated against *V. anguillarum* and *A. salmonicida.* However, the effectiveness of the vaccines is still under review as the

variable nature of isolated bacterial strains may complicate the vaccine development (Hjeltnes B and Walde 2017, Gulla et al. 2015).

2. Objectives

The main objective of this dissertation is to characterize immune and stress factors in skin/skin mucus of the cleaner fish, *Cyclopterus lumpus* under various physiological conditions. Specific objectives that are focused during the study are

1. To establish a skin mucus proteome using 2D gel based proteomics (**paper I**).

2. To find the proteomic and histological differences in lumpfish skin among the dorsal, caudal and ventral regions (**paper II**)

3. To study the effect of chronic stress on lumpfish skin proteome using 2D gel based proteomics and mRNA expression levels of selected stress/immune relevant genes (**paper III**)

4. To study the effect of chronic stress on hypothalamus-pituitary-interrenal axis of lumpfish (**paper IV**).

5. To study the effect of two different temperatures on lumpfish skin proteome using gel based proteomics and the transcriptome using RNA sequencing (**paper V**).

3. Summary of papers

Paper I

Skin mucus proteins of Lumpsucker (*Cyclopterus lumpus*) skin mucus

Biochemistry and biophysics reports, 2017; 9:217-25

In this paper we used two-dimensional gels and tandem mass spectrometry to identify skin mucus proteins in lumpsucker skin. Paper I highlights the presence of several proteins in skin mucus of lumpfish that have possible immune and/or stress related functions. According to gene ontology analysis the proteins were involved in several biological processes 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:0002376), localisation (GO:0051179), metabolic process (GO:0008152), multicellular organismal process (GO:0032501), and response to stimulus (GO:0050896). The identified proteins have also been reported in skin mucus of other teleost species.

Paper II

Proteomic and structural differences in lumpsucker skin among dorsal, caudal and ventral region

Manuscript submitted

Lumpsucker belongs to the family Cyclopteridae. It has a globe shaped body with several ridges and scutes all over the body. The head is small and followed by a hump like structure on the dorsal part of the body. At the ventral region, the pelvic fin of this fish is modified into an adhesive disc. We conducted this study to find the differences in protein expression and skin structure among various regions of the lumpsucker body. We sampled skin from three regions of the body; i) dorsal region near the operculum ii) caudal region

near the caudal peduncle iii) ventral region near the adhesive disc. We used a classical two dimensional gel based method for the protein expression part. We identified cytoskeletal, antimicrobial, immune and stress response proteins that were differentially expressed among the three regions. The results showed that most of the proteins had a relatively high expression level in the ventral region compared to the dorsal and caudal regions. To study the structural differences among the three regions we used alcian blue (pH 2.5) and periodic acid Schiff stained skin sections. The findings showed differences in epidermal thickness and goblet cell counts between the three regions. The ventral region showed high goblet cell count and thicker epidermis compared to dorsal and caudal region. In the skin micrographs we observed several cells similar to saccular cells found in other teleost species. These saccular like cells were absent in the ventral region of the skin. A few immune and/or stress related genes were selected to find the mRNA expression level among the three regions. The protein expression differences did not correlate with the mRNA expression levels of the genes. Additionally, we established a proteome reference map of naïve lumpsucker skin by identifying abundant proteins in lumpsucker skin.

Paper III

Effect of chronic stress on skin proteome of lumpfish, *Cyclopterus lumpus*.

Manuscript

Stress is an inevitable part in intensive fish farming. Study of different stressors and their impact on fish health is needed for successful production and fish welfare. In this manuscript we study the effect of prolonged crowding stress on lumpfish skin. The stress was induced by lowering the water level and refilling it after approximately 20 min once everyday for a period of 28 days. We sampled skin tissues on day 7, 14, 21 and 28 for proteomics analysis using two-dimensional gels and tandem mass spectrometry. Several proteins were differentially expressed in the stressed group due to the stress. The proteins are calmodulin, guanine nucleotide binding protein subunit beta 2, glutathione-S-

transferase Mu 3, fatty acid binding protein, heat shock cognate 70 kDa protein, keratin, histone H4 and 14-3-3 alpha/beta. In this study we also performed real time quantitative PCR to find the expression of the *hspa8* gene. This gene was differentially expressed and the expression level was high in the stressed group compared to control on day 7, but it did not show significant differences on day 14, 21 and 28.

Paper IV

The effect of chronic stress on primary, secondary and tertiary stress responses and its impact on animal welfare in lumpfish.

Manuscript

This manuscript investigated the effect of chronic stress (from the same experiment as in paper III) on the hypothalamic-pituitary-interrenal (HPI) axis and the basal level of cortisol in lumpfish. Blood samples were obtained on pre-stress, 7, 14, 21, and 28 day to assess the changes in cortisol levels. The response on the HPI axis was studied by stimulation (adrenocorticotropic hormone) and suppression (dexamethasone) tests. The results indicated an allostatic overload type 2 due to oversensitivity of ACTH. It also showed a reduced negative feedback system and elevated cortisol level in the plasma. As a secondary stress response the stressed fishes showed an imbalance in magnesium ion concentration, osmolality and chloride levels. The fishes from the stressed group showed reduced growth indicating the tertiary effect of chronic stress. The findings from this study indicating that lumpfish welfare will be compromised if the stress becomes chronic as it fails to regain the homeostasis resulting in allostatic overload type 2.

Paper V

A multi-omics approach to assess the effect of temperature on lumpsucker (*Cyclopterus lumpus*) skin

Manuscript

In this study we investigated the effect of temperature acclimation on lumpfish skin. The fishes were held at two temperatures, 8°C (cold acclimation, normal holding temperature) and 14°C (warm acclimation) for a period of 7 days to study the changes in skin molecules due to acclimation. We used two dimensional gel based proteomics and a illumina NextSeq RNA sequencing method to find differentially expressed proteins and genes. The differentially expressed proteins and genes are small ubiquitin-related modifier 2, prefoldin subunit 5, SH3 domain-binding glutamic acid-rich-like protein, protein mago nashi homolog, proteasome subunit beta type 1-A, retinoblastoma binding protein 4-like, angiotensin-like, microfibril-associated glycoprotein 4-like, apolipoprotein E, 40S ribosomal protein, annexin A3 and catechol methyltransferase domain- containing protein 1-like protein, thioredoxin interacting gene, phosphate and actin regulator, disintegrin and metallopeptidase with thrombospondin motif, metallopeptidase with thrombospondin type-1, proline synthase co-transcribed homolog, atherin and catenin delta 2. The differentially expressed proteins and genes were involved in cytoskeletal organization, energy metabolism, protein folding and biosynthesis.

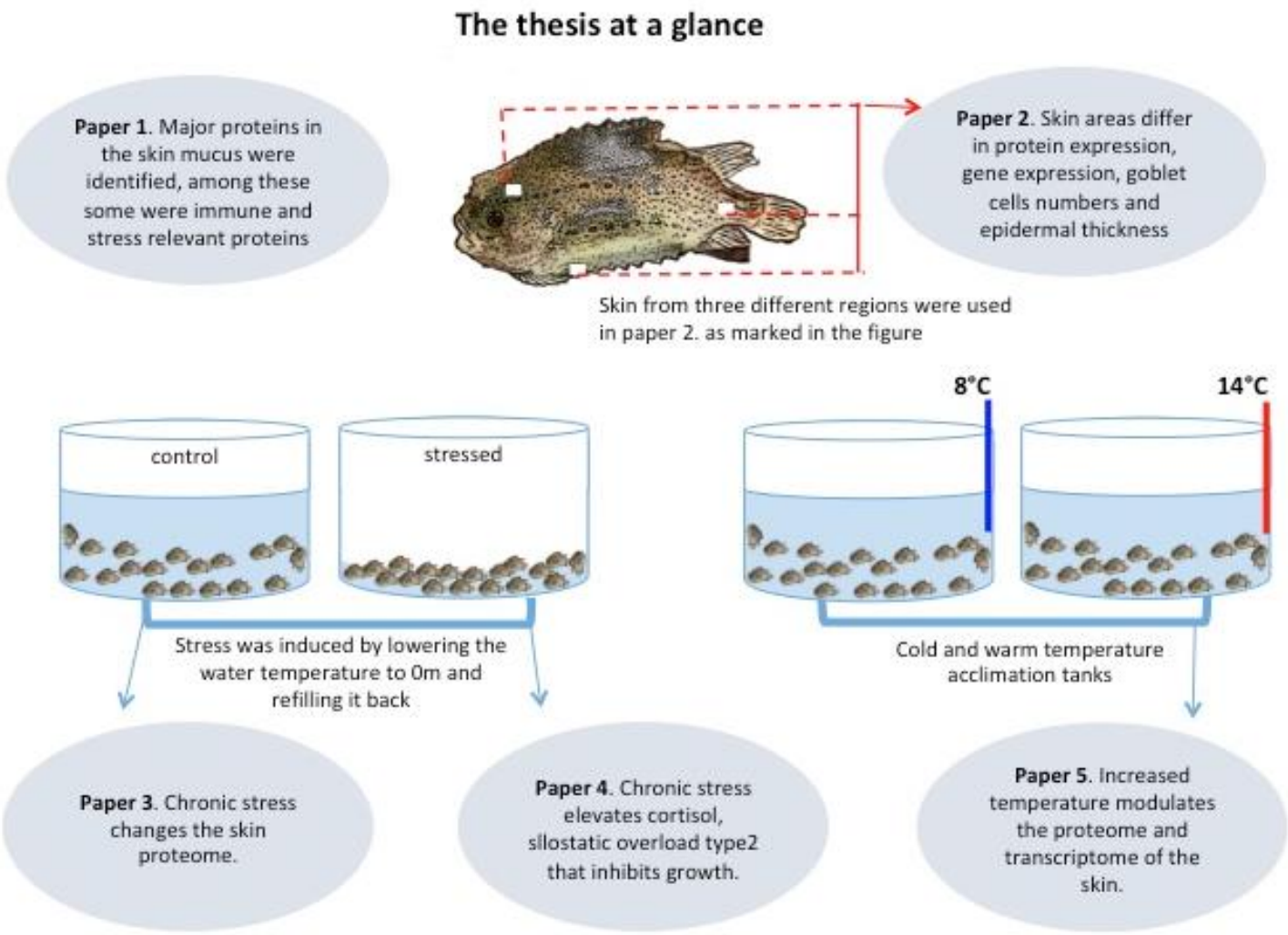

Figure 4. Graphical view of the main results of the experiments

4. General discussion

This dissertation is the first report on characterization of lumpfish skin and skin mucus proteins using gel based proteomics (**paper I, II, III and V**). The present study identified lumpfish skin proteins and their differences in expression levels among three different body sites; the dorsal region above operculum (D), the caudal region close to caudal peduncle (C) and the ventral region near the adhesive disc (V) using two dimensional gel electrophoresis and LC-MS/MS. The structural differences among D, C and V were studied using Periodic Acid Schiff and alcian blue stained skin sections (**paper II**). Further, comparison based studies were performed to explore the physiological responses induced by chronic stress and temperature change in lumpfish skin (**paper III, IV and V**).

Stress is an integral part in intensive fish farming. Crowding, handling, transportation, changes in water parameters such as temperature, ammonia, salinity and oxygen level are common stressors in aquaculture systems. We measured plasma cortisol, lactate, glucose, osmolality, chloride levels and performed the stimulation and suppression test of HPI axis to study the response of hypothalamic-pituitary-interrenal (HPI) axis of lumpfish subjected to long term crowding stress of 28 days (**paper IV**). From the same experiment, the changes in protein expressions in skin of lumpfish due to crowding were studied using two dimensional gel electrophoresis and LC- MS/MS (**paper III**). Changes in protein and gene expressions in lumpfish skin kept at low (8 °C) and high (14 °C) temperature were studied using gel based proteomics and RNA-sequencing (**paper V**).

4.1. Lumpfish skin structure

Lumpfish has an interesting body shape with a short head, hump-like dorsal crest, tubercles and ridges all over the body, and the pelvic fin modified into an adhesive disc (Davenport 1985). These features lead us to study the skin structure of lumpfish and also the differences between regions D, C and V (**paper II**). The skin showed differences in

goblet cell counts, epidermal thickness and presence of saccular cells among the three regions. Relatively high numbers of goblet cells were observed in the ventral region compared to dorsal and caudal regions (**paper II**). Differences in goblet cell density and size among different body sites were also observed in Atlantic salmon skin. The dorsolateral region of skin had significantly larger and denser goblet cells compared to head region (Pittman et al. 2013).

In **paper II** we measured the thickness of epidermis from dorsal, ventral and caudal region. The epidermis of the ventral region was thicker compared too the dorsal and caudal regions. Similar results were obtained in gilthead sea bream and a few benthic species (Cordero et al. 2017b, Elliott 2000). Thicker epidermis in the ventral region of lumpfish might be good to provide mechanical protection while swimming or attaching to surrounding objects. When lumpfish attaches to surfaces with biofilm, thicker skin might give protection from pathogen making the penetration more difficult. Lumfish being a scaleless fish, thicker epidermis might provide protection in absence of scales against rubbing or chaffing with rocky surfaces. We also observed cells similar to saccular cells/sacciform cells in dorsal and caudal region of skin but not in ventral region. These cells have been observed in several teleost species such as Atlantic halibut, channel catfish (*Ictalurus punctatus*), brown trout (*Salmo trutta*) and Arctic char (*Salvelinus alpinus*) (Ottesen and Olafsen 1997, Pickering and Fletcher 1987). Pickering and Fletcher, 1987 suggested that sacciform cells in salmonids secrete a proteinaceous material that could protect the fish from infections. However, in lumpfish these cells were found empty that could be due to the Alcian blue (pH- 2.5) staining used in this study (Mittal et al. 1994).

4.2. Skin proteins/genes of lumpfish and their possible roles in immunity and stress response

Lumpfish is a non-model species that neither has its genome published nor have any reference protein or gene sequences in public databases. To obtain knowledge about its proteins/genes involved in skin immunity we used homology driven gel based proteomics, quantitative real time PCR and illumina RNA-seq analysis. We identified several proteins/genes (**paper I, II, III, and V**) both in skin and skin mucus that have possible roles in stress and/or immune response in fish. All proteins/genes identified in **paper I, II, III, and V** are grouped as proteins/genes involved in immune response, stress response, cell communication, protein metabolism, lipid metabolism, carbohydrate metabolism, cytoskeletal organization, nucleic acid metabolism and binding. The proteins/genes were grouped as their gene ontology biological process (retrieved from UniProt and literature available) as mentioned in **paper I, II, III and V**. Not all identified proteins/genes have direct roles in immune or stress response, but they could have roles indirectly in fish immunity and coping with stress.

Immune response proteins/genes identified were natural killer enhancing factor/peroxiredoxins, pentraxin, cystatin B, glutathione-S-transferase, annexin-A1 and A3 and thioredoxin interacting gene. Natural killer enhancing factor or peroxiredoxins antioxidants have roles in inflammation and innate immune responses against pathogenic bacteria and virus in teleosts (Esteban et al. 2013). Thioredoxin interacting gene are an essential part of thioredoxin system, essential in regulation of oxidative stress response (Patwari et al. 2006, Falfushynska et al. 2016) Pentraxins are lectin like proteins that could have roles in fish innate immunity (Gupta 2012). Annexin-3 was upregulated in lumpfish skin in warm acclimation (**paper V**). In human this protein has been found in neutrophils suggesting their possible immune roles. Annexin-1 was identified in skin

mucus of lumpfish. This protein is exported to the extracellular space by non-classical transport (Wein et al. 2004) and not by exocytosis as it lacks a signal peptide (Christmas et al. 1991).

Proteins/genes involved in stress responses identified in this study are heat shock cognate 70 kDa, heat shock cognate 71kDa (also known as heat shock protein 70kDa) and warm temperature acclimation protein. Heat shock proteins are well known stress indicators (Tomanek and Sanford 2003, Iwama et al. 2004). In the chronic stress experiment (**paper III**) heat shock cognate 70kDa protein was differentially expressed at all time points (7, 14, 21 and 28 days). The heat shock cognate 70 kDa gene showed differential expression in the mRNA level in the skin in naïve fish among different region of skin (**paper II**) and in skin of chronically stressed fish (**paper III**). Warm temperature acclimation protein was initially identified in goldfish and named so because of its expression change due to temperature differences (Kikuchi et al. 1997). This protein have been suggested be involved in immune responses in channel catfish infected with *Edwardsiella ictaluri.* Up regulation of this protein upon infection in channel catfish could be to eliminate the bacterial infection by binding to heme and making it unavailable for bacteria which is essential for its growth and colonization (Sha et al. 2008).

Proteins/genes involved in cell communication such as calmodulin, 14-3-3 and rhophilin were identified in this study. Calmodulin was identified in skin mucus and skin of naive fish (**paper I, II and III**) and differentially expressed in skin of stressed fish on days 14 and 21 due to prolonged crowding stress. This is a highly conserved calcium binding protein involved in cell signalling, immune and stress responses (Berchtold and Villalobo 2014). Several spots in skin mucus were identified as 14-3-3 protein (**paper I**). It is a signalling protein that was found to have role in phagocytosis and resistance against microbial infection in zebrafish (Ulvila et al. 2011). 14-3-3 beta/alpha protein was differentially expressed in lumpfish skin under prolonged crowding (**paper III**). This protein

was associated with osmoregulatory signal transduction in gill epithelium of a small killifish (*Fundulus heteroclitus*) (Kültz et al. 2001). Regulation of this protein in stressed fishes could suggest its role in osmoregulation in lumpfish. Extracellular 14-3-3 can modulate matrix metalloproteinase-1 in human lungs and are suggested to influence collagen breakdown (Asdaghi et al. 2012). Extracellular 14-3-3 has been shown to have roles wound healing, fibrosis, rheumatoid arthritis, Creutzfeldt-Jakob disease and lung airway remodeling (reviewed in (Kaplan et al. 2017)) in human.

Proteins involved in protein metabolism identified in (**paper I, II and V**) were ubiquitin, proteasome subunits, protein disulphide isomerases Ubiquitin in mammals are involved in stress responses (Gubellini et al. 1997, Yamamoto et al. 2006). When coping with changes protein remodelling can occur, both protein synthesis and protein degradation will thus take place. Ubiquitin are targeting proteins for degradation in the proteasomes. Protein disulphide isomerases are involved in protein folding.

Proteins identified in (paper I, II, V) which are involved in lipid metabolism are apolipoproteins and fatty acid binding protein. Apolipoproteins have been found in skin mucus and skin of many teleosts (Concha et al. 2003, Pridgeon and Klesius 2013, Rajan et al. 2013b including lumpfish (**paper I, II, V**) in this study (Concha et al. 2003, Pridgeon and Klesius 2013, Rajan et al. 2013b). Apolipoprotein A1 showed lytic activity against fish pathogens in channel catfish suggesting their role in fish defence (Pridgeon and Klesius 2013). Apolipoprotein increased expression in skin mucus of Atlantic cod after natural infection with *Vibrio anguillarum* (Rajan et al. 2013b). In contrast to this it was downregulated in skin mucus of gilthead seabream after chronic wounds were induced and let to start healing (Cordero et al. 2017a).

Proteins involved in carbohydrate metabolism identified (**paper I and I**I) were nucleoside diphosphate kinase B, alpha enolase, glyceraldehyde-3-phosphate

dehydrogenase and triosephosphate isomerases. Among these proteins, alpha enolase was found to have relatively high expression in longjaw mudsucker (*Gillicthys mirabilis*) during hypoxic condition that might serve as stress marker in fish (Gracey et al. 2001).

Proteins/genes involved in cytoskeletal organization, skeletal system development and muscle contraction were identified in lumpfish skin (**paper I, II and V**). These are keratin, actin, tubulin, myotrophin, glial fibrillary acidic protein, septin, coactosin, collagen, cofilin, microfibrill associated glycoprotein 4 like and myosin. The genes identified in **paper V** were A disintegrin and metalloproteinase with thrombospondin motif 8, metallopeptidase with thrombospondin motifs, catenin delta 2 and phosphate and actin regulator 1. Apart from the role in cytoskeletal organisation these proteins/genes are also involved in immune responses. A glycoprotein identified in rainbow trout skin mucus similar to keratin type II showed anti-microbial activity by its pore forming activity (Molle et al. 2008). Actin is essential for phagocytosis. Cofilin-1's role in F-actin modulation makes it a key molecule in controlling cell mobility including chemotaxis. The genes/proteins identified in paper V might be involved in cytoskeletal reorganization that was effected due to temperature acclimation. The A disintegrin and metalloproteinase with thrombospondin motif was upregulated due to heat stress in catfish showing its role in stress response (Liu et al. 2013), phosphate and actin regulator genes are involved in actin filament rearrangement (Allen et al. 2004), catenins are involved in cell-cell adhesion (Paffenholz and Franke 1997, Anastasiadis and Reynolds 2000).

Proteins involved in nucleic acid metabolism, binding and nucleosome complex formation identified (**paper I, II, III and V**) were ATP synthase subunit beta, guanine nucleotide binding protein, adenylate kinase, DNA binding protein RFX-2, ribosomal proteins and histone proteins (histone H2A, H2B, H4 and H3.2). Histone proteins, apart from their role in the nucleosome complex are known for their antimicrobial activities in

fish (Fernandes et al. 2002, Parseghian and Luhrs 2006). Histone H4 was differentially expressed in lumpfish skin under crowding stress (**paper III**) and showed relatively high expression in ventral region of skin compared to dorsal and caudal in healthy fish (**paper II**).

Other important proteins in lumpfish skin identified (**paper I and II**) are natterin-2, transferrin, serotransferrin, and parvalbumin. Natterin is a mannose binding lectin with possible roles in complement pathway activation and subsequent immune response (Rajan et al. 2017b). Transferrin and serotransferrin could limit the availability of iron for bacteria that inhibits growth and colonization. This protein is also involved in macrophage activation (Stafford and Belosevic 2003).

In paper **III** the mRNA expression level was assessed using quantitative real time PCR to find out their presence locally. The mRNA expression in the current study did not follow the protein expression such as heat shock cognate 70 kDa protein and mRNA expression level in **paper III.** In **paper II** apolipoprotein A1 and histone H2B genes were differentially expressed whereas the protein did not show any differential expression. Similar results were obtained in Atlantic cod challenged with *Vibrio anguillarum* (Rajan et al. 2013b) and a study on yeast *Saccharomyces cervisiae* (Gygi et al. 1999) where the protein and RNA expression did not correspond with each other. Some of the studies have been performed on yeast, *Esherchia coli* and mouse (*Mus musculus*) to investigate the protein and mRNA correlation where the authors found a low Perason's correlation coefficient (r_p) such as 0.39 in *Saccharomyces cervisiae* (Gygi et al. 1999), 0.57 in *Escherchia* coli (Ishihama et al. 2005) and 0.59 in *Mus musculus* (Tian et al. 2004). Some of the factors affecting the protein – mRNA correlation may be the RNA secondary structure, protein half lives, binding of regulatory proteins to mRNA that stops translation, codon bias, ribosomal density and ribosome occupancy (Maier et al. 2009).

4.3. Methodological aspects

Several methods were used to achieve the objectives of this study. The methods include tissue sampling and protein extraction, and identification of proteins in skin and its mucus by homology driven gel based proteomics (**paper I, II, III and V**), skin histology (**paper II**), methods to assess stress level in fish such as plasma cortisol, glucose, lactate, osmolality, chloride levels, stimulation and suppression test of HPI axis (**paper IV**), RNA sequencing and *de novo* assembly for analysis of skin transcriptome (**paper V**), degenerate PCR (**paper II**) and real time quantitative PCR (**paper II and III**).

In the present study, skin mucus (**paper I**) and skin tissue (**paper II, III and V**) from lumpfish was used to analyze its proteomic, transcriptomic and histological aspects under different physiological conditions. Skin mucus was sampled by massaging the fish for a few seconds while keeping it inside a plastic bag. The mucus was then collected using a spatula and filled in tubes. Massaging the fish for few seconds (25-30s) stimulated the mucus secretion for collection of a fair amount of mucus for further analysis. Skin mucus can be sampled by putting the fish in a plastic bag with suitable buffer (Easy and Ross 2009) or scrapping the skin with a sterile glass slide or spatula (Rajan et al. 2011). Both the methods have pros and cons, using plastic bags increase the risk of fecal contamination whereas using a spatula or glass slide avoids the risk of contamination but did not provide enough samples to carry out the analysis. Any contamination in the sample could be removed by centrifugation/filtration to remove debris (Reddy et al. 2004). In the case of lumpfish sampling of mucus, using a plastic bag and a little massage was found advantageous over scrapping with a glass slide due to its rough skin with scutes and ridges.

Homology driven two dimensional gel electrophoresis coupled with LC-MS/MS (liquid chromatography- mass spectrometry) is a robust and suitable method for analyzing proteomes in comparative studies of species like lumpfish with minimum data available on

public databases (Rogowska-Wrzesinska et al. 2013). We used this method for protein expression analysis in **paper I, II, III, and V**. It separates the proteins in two dimensions. First, according to its isoelectric point and then it is separated according to its molecular weight. The aid of sensitive ruthenium based fluorescent stain such as Sypro Ruby® gives a better resolution and detection of proteins as little as 1 ng compared to stains like Coomassie blue and silver stain (Berggren et al. 2000). Sypro Ruby® is linear over three orders of magnitude range (Lopez et al. 2000), this is important for studies comparing spot or band densities. However, gel based methods do not give a deep coverage of the proteome compared to gel free proteomics because of certain limitations. 2D gel based proteomics cannot identify protein that have apI more than 10 and less than 3 because the isoelectric focusing does not perform well outside the range of pI 3-10. Also proteins of very high molecular weights are not suitable for separation in gel based methods due to problem in solubilization of large proteins (Rogowska-Wrzesinska et al. 2013). Hydrophobic proteins are also problematic when one use gel based proteomics, as they will not be dissolved well or precipitate in the first dimension. A study comparing gel-free and gel-based proteomics concluded that the two approaches identify complementary rather overlapping proteins and hence both methods have their place in obtaining information on proteomes (Jurado et al. 2015).

Histology of skin sections was performed to study the structure of lumpfish skin (**paper II**). We used alcian blue (pH-2.5) and periodic acid Schiff's staining in this study. It is a suitable staining technique used for goblet cell staining. The alcian blue stains the acid mucins deep blue and periodic acid Schiff's stains the neutral mucins magenta. Cells containing both acid and neutral mucins become dark blue or purple in color as in **paper II**. The cells similar to saccular cells did not show any cell content this could be an artifact introduced by the Alcian blue (pH 2.5) staining as similar results were obtained by Mittal et al, 1994.

Degenerate primers were used in this study to amplify specific immune and/or stress related genes (**paper II**). In species like lumpfish without any sequences in public databases, designing degenerate primers is a suitable method for downstream analysis. However, successful amplification using degenerate primers depends on the degree of degeneracy. The lower the degeneracy the higher the chances of successful amplification. In this study only a few of the genes did successfully amplify.

Gene expression was performed using a RNA-seq method by using a *de novo* assembly method in the absence of a lumpfish genome (**paper V**). This method allows for a deeper coverage of the transcriptome, with precise level of transcript and isoform measurements (Wang et al. 2009). We used Trinity *de novo* assembler in this study. Trinity is a widely used program for transcriptome assembly for non model species without an available reference genome as it recovers the full length transcripts across a broad range of expression, compared to other assemblers (Grabherr et al. 2011). Further studies could use the genomic resources from the RNA-seq analysis (**paper V**).

5. Conclusions

- We identified several key proteins in lumpfish skin and its mucus that are involved in stress and immune responses in fish and other vertebrates.
- The skin epidermal layer in the ventral region is relatively thicker and have relatively high numbers of goblet cells compared to the dorsal and caudal regions. Saccular cells were observed in the dorsal and caudal skin sections, but not in the ventral region.
- Protein expression differs between different body sites in lumpfish. The skin in the ventral region near the adhesive disc has relatively high levels of proteins such as collagen and parvalbumin compared to the dorsal and caudal region.
- Changes in protein expression were observed in the skin in the prolonged crowding stress experiment. Differentially expressed proteins with immune roles were calmodulin, fatty acid binding protein, histone H4, keratin, guanine nucleotide binding protein, glutathione-S-transferase, heat shock cognate 70kDa and 14-3-3 beta/alpha protein. Heat shock cognate 70kDa showed significant difference at all time points 7, 14, 21, and 28 days, which suggest it could serve as a stress marker in lumpfish skin.
- Daily crowding stress compromised the fish welfare. The results indicated that the stress group showed signs of allostatic overload type 2 (chronic stress) due to oversensitivity to adrenocorticotropic hormone (ACTH) and a reduced negative feedback system, with increased baseline levels of cortisol as a result. These chronic changes in the endocrine system promoted changes in the secondary and the tertiary stress responses as reduced osmoregulatory capacity and stunted growth. These results show that the robust lumpfish can suffer from chronic stress with possible dire consequences for the animal welfare.
- Lumpfish skin showed few changes in the transcriptome level when subjected to low (8 °C) and high (14 °C) temperature. The proteomic analysis identified

differentially expressed immune, cytoskeletal and metabolism related proteins such as apolipoprotein E, annexin 3, prefoldin subunit 5, small ubiquitin related modifier protein, SH3 domain binding glutamic acid rich like protein, protein mago nashi homolog, proteasome subunit beta type 1, angiotensinogen like, microfibril associated glycoprotein 4 like and catechol methyltransferase domain containing protein 1 like protein. Combining the protein and gene expression the results indicated that lumpfish skin is moderately influenced by shifting the temperature from 8 °C to 14 °C.

Over all, this thesis improved the understanding of lumpfish skin structure and the skin and skin mucus molecular components focusing on immune and stress responses. The set of proteins and genes identified in this study could serve as a benchmark for a wide range of physiological comparisons and in-depth functional studies of stress and immune relevant proteins/genes.

6. Future perspectives

The current study provides an insight into proteins/genes in the lumpfish skin and skin mucus with possible immune functions. Although skin immunity in fish is not a new topic for discussion, still there are many aspects that have to be addressed. The fishes in general do have similarities in their functioning of the immune system but as “not one size fits all”, they do have unique features. Therefore, study of individual species and their biology is important for a sound understanding of fish defence mechanisms. Here are a few directions for further studies:

- In the comparison studies it would be informative to include sex as a factor to know the difference of stress levels between male and female. This would be helpful in maintaining healthy brood stocks.
- Further investigation on saccular cells and their function in lumpfish could aid in the understanding of skin structure and function of lumpfish.
- Production of antibodies against for example heat shock cognate 70 kDa, apolipoprotein, calmodulin and histone proteins could be useful to study the immune functions in depth in lumpfish.
- Proteins such as apolipoprotein, calmodulin and natterin-2 have been identified in both skin mucus and skin of lumpfish. Characterization of these proteins to unveil their extracellular functions in the fish skin and skin mucus could be useful to understand their role in stress responses.
- The generated data (protein and gene sequences) could be exploited for future studies comparison studies targeting molecular factors.
- Development of lumpfish skin organ culture or cell lines could reduce the usage of live animals and could be used for various research topics, e.g. infection studies.

7. References

Alam SN, Yammine H, Moaven O, Ahmed R, Moss AK, Biswas B, et al. (2014). Intestinal alkaline phosphatase prevents antibiotic-induced susceptibility to enteric pathogens. Ann Surg 259**:** 715-722.

Alarcón M, Gulla S, Røsæg MV, Rønneseth A, Wergeland H, Poppe TT, et al. (2016a). Pasteurellosis in lumpsucker *Cyclopterus lumpus*, farmed in Norway. J Fish Dis 39**:** 489-495.

Alarcón M, Thoen E, Poppe TT, Bornø G, Mohammad SN & Hansen H (2016b). Co-infection of Nucleospora cyclopteri (Microsporidia) and Kudoa islandica (Myxozoa) in farmed lumpfish, Cyclopterus lumpus L., in Norway: a case report. J Fish Dis 39**:** 411-418. DOI 10.1111/jfd.12372

Alibardi L & Joss JMP (2003). Keratinization of the epidermis of the Australian lungfish Neoceratodus forsteri (dipnoi). J Morphol 256**:** 13-22. DOI 10.1002/jmor.10073

Allen PB, Greenfield AT, Svenningsson P, Haspeslagh DC & Greengard P (2004). Phactrs 1–4: A family of protein phosphatase 1 and actin regulatory proteins. P Natl Acad Sci USA 101**:** 7187-7192.

Altringham JD & Block BA (1997). Why do tuna maintain elevated slow muscle temperatures? Power output of muscle isolated from endothermic and ectothermic fish. J Exp Biol 200**:** 2617.

Anastasiadis PZ & Reynolds AB (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. J Cell Sci 113**:** 1319.

Aranishi F, Mano N & Hirose H (1998). Fluorescence localization of epidermal cathepsins L and B in the Japanese eel. Fish Physiol Biochem 19**:** 205-209. DOI 10.1023/A:1007779600183

Åsbakk K (2001). Elimination of foreign material by epidermal malpighian cells during wound healing in fish skin. J Fish Biol 58**:** 953-966. DOI 10.1111/j.1095-8649.2001.tb00547.x

Asdaghi N, Kilani RT, Hosseini-Tabatabaei A, Odemuyiwa SO, Hackett T-L, Knight DA, et al. (2012). Extracellular 14-3-3 from human lung epithelial cells enhances MMP-1 expression. Mol Cell Biochem 360**:** 261-270. DOI 10.1007/s11010-011-1065-1

Bañón R, Garazo A & Fernández A (2008). Note about the presence of the lumpsucker Cyclopterus lumpus (Teleostei, Cyclopteridae) in Galician waters (NW Spain). J Appl Ichthyol 24**:** 108-109. DOI 10.1111/j.1439-0426.2007.00839.x

Barton BA (2002). Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids1. Integrativ Comp Biol 42**:** 517-525. DOI 10.1093/icb/42.3.517

Belanger JM, Son JH, Laugero KD, Moberg GP, Doroshov SI, Lankford SE, et al. (2001). Effects of short-term management stress and ACTH injections on plasma cortisol levels in cultured white sturgeon, Acipenser transmontanus. Aquaculture 203**:** 165-176. DOI

Berchtold MW & Villalobo A (2014). The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer. BBA Mol Cell Res 1843**:** 398-435. DOI

Berggren K, Chernokalskaya E, Steinberg TH, Kemper C, Lopez MF, Diwu Z, et al. (2000). Background-free, high sensitivity staining of proteins in one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gels using a luminescent ruthenium complex. Electrophoresis 21**:** 2509-2521. DOI

Bly JE & Clem LW (1992). Temperature and teleost immune functions. Fish Shellfish Immunol 2**:** 159-171. DOI

Chivers DP, Wisenden BD, Hindman CJ, Michalak TA, Kusch RC, Kaminskyj SGW, et al. (2007). Epidermal 'alarm substance' cells of fishes maintained by non-alarm functions: possible defence against pathogens, parasites and UVB radiation. P Roy Soc B Biol Sci 274**:** 2611-2619. DOI 10.1098/rspb.2007.0709

Christmas P, Callaway J, Fallon J, Jones J & Haigler HT (1991). Selective secretion of annexin 1, a protein without a signal sequence, by the human prostate gland. J Biol Chem 266**:** 2499-2507.

Chrousos GP (1998). Stressors, Stress, and Neuroendocrine Integration of the Adaptive Response: The 1997 Hans Selye Memorial Lecture. Ann NY Acad Sci 851**:** 311-335. DOI 10.1111/j.1749-6632.1998.tb09006.x

Concha MI, Molina SA, Oyarzún C, Villanueva J & Amthauer R (2003). Local expression of apolipoprotein A1 gene and a possible role for HDL in primary defence in the carp skin. Fish Shellfish Immunol 14**:** 259-273.

Cordero H, Brinchmann MF, Cuesta A & Esteban MA (2017a). Chronic wounds alter the proteome profile in skin mucus of farmed gilthead seabream. BMC Genomics 18**:** 939. DOI 10.1186/s12864-017-4349-3

Cordero H, Brinchmann MF, Cuesta A, Meseguer J & Esteban MA (2015). Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). Proteomics. DOI 10.1002/pmic.201500120

Cordero H, Ceballos-Francisco D, Cuesta A & Esteban MÁ (2017b). Dorso-ventral skin characterization of the farmed fish gilthead seabream (*Sparus aurata*). PloS One 12**:** e0180438.

Cordero H, Morcillo P, Cuesta A, Brinchmann MF & Esteban MA (2016). Differential proteome profile of skin mucus of gilthead seabream (*Sparus aurata*) after probiotic intake and/or overcrowding stress. J Proteomics 132**:** 41-50.

Davenport J (1985). Synopsis of biological data on the lumpsucker, *Cyclopterus lumpus* (Linnaeus, 1758)*,* Rome, Italy.

Davenport J & Thorsteinsson V (1989). Observations on the colours of lumpsuckers, Cyclopterus lumpus L. J Fish Biol 35**:** 829-838. DOI 10.1111/j.1095-8649.1989.tb03034.x

Dhabhar FS (2009). Enhancing versus Suppressive Effects of Stress on Immune Function: Implications for Immunoprotection and Immunopathology. Neuroimmunomodulat 16**:** 300-317. DOI 10.1159/000216188

Dhabhar FS & Mcewen BS (1999). Enhancing versus suppressive effects of stress hormones on skin immune function. P Natl Acad Sci USA 96**:** 1059-1064.

Easy RH & Ross NW (2009). Changes in Atlantic salmon (*Salmo salar*) epidermal mucus protein composition profiles following infection with sea lice (*Lepeophtheirus salmonis*). Comp Biochem Phys D 4**:** 159-167.

Elliott DG (2000). Microscopic functional anatomy: Integumentary system: Chapter 17. The laboratory fish**:** 271-306. DOI 10.1016/B978-012529650-2/50023-8

Esteban MA (2012). An Overview of the immunological defenses in fish skin. ISRN Immunol 2012**:** 1-29.

Esteban MA, Chaves-Pozo E, Arizcun M, Meseguer J & Cuesta A (2013). Regulation of natural killer enhancing factor (NKEF) genes in teleost fish, gilthead seabream and European sea bass. Mol Immunol 55**:** 275-282.

Esteban MÁ & Rebeca C (2015). 4 - Fish mucosal immunity: skin A2 - Beck, Benjamin H. In Peatman, E. (Ed.) Mucosal Health in Aquaculture. San Diego: Academic Press.

Faílde LD, Bermúdez R, Vigliano F, Coscelli GA & Quiroga MI (2014). Morphological, immunohistochemical and ultrastructural characterization of the skin of turbot (Psetta maxima L.). Tissue Cell 46**:** 334-342.

Falfushynska HI, Phan T & Sokolova IM (2016). Long-Term Acclimation to Different Thermal Regimes Affects Molecular Responses to Heat Stress in a Freshwater Clam Corbicula Fluminea. Sci Rep 6**:** 39476. DOI 10.1038/srep39476

Fast MD, Sims DE, Burka JF, Mustafa A & Ross NW (2002). Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comp Biochem Physiol A Mol Int Physiol 132**:** 645-657.

Fernandes JMO, Kemp GD, Molle MG & Smith VJ (2002). Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Biochem J 368**:** 611-620.

Fishelson L (1984). A comparative study of ridge-mazes on surface epithelial cell-membranes of fish scales (Pisces, Teleostei). Zoomorphology 104**:** 231-238. DOI 10.1007/BF00312036

Freitas C, Olsen EM, Moland E, Ciannelli L & Knutsen H (2015). Behavioral responses of Atlantic cod to sea temperature changes. Ecol Evol 5**:** 2070-2083. DOI 10.1002/ece3.1496

Gandar A, Laffaille P, Canlet C, Tremblay-Franco M, Gautier R, Perrault A, et al. (2017a). Adaptive response under multiple stress exposure in fish: From the molecular to individual level. Chemosphere 188**:** 60-72.

Gandar A, Laffaille P, Marty-Gasset N, Viala D, Molette C & Jean S (2017b). Proteome response of fish under multiple stress exposure: Effects of pesticide mixtures and temperature increase. Aquat Toxicol 184**:** 61-77.

Glover CN, Bucking C & Wood CM (2013). The skin of fish as a transport epithelium: a review. J Comp Physiol B 183**:** 877-891. DOI 10.1007/s00360-013-0761-4

Gomez D, Sunyer JO & Salinas I (2013). The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. Fish Shellfish Immunol 35**:** 1729-1739.

Goymann W & Wingfield JC (2004). Allostatic load, social status and stress hormones: the costs of social status matter. Anim Behav 67**:** 591-602.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29**:** 644.

Gracey AY, Troll JV & Somero GN (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. P Natl Acad Sci USA 98**:** 1993-1998.

Guardiola FA, Cuesta A, Arizcun M, Meseguer J & Esteban MA (2014). Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (Sparus aurata). Fish Shellfish Immunol 36**:** 545-551.

Guardiola FA, Cuesta A & Esteban MÁ (2016). Using skin mucus to evaluate stress in gilthead seabream (Sparus aurata L.). Fish Shellfish Immunol 59**:** 323-330.

Gubellini P, Bisso GM, Ciofi-Luzzatto A, Fortuna S, Lorenzini P, Michalek H, et al. (1997). Ubiquitin-Mediated Stress Response in a Rat Model of Brain Transient Ischemia/Hypoxia. Neurochem Res 22**:** 93-100. DOI 10.1023/A:1027389623767

Gulla S, Sørum H, Vågnes Ø & Colquhoun DJ (2015). Phylogenetic analysis and serotyping of Vibrio splendidus-related bacteria isolated from salmon farm cleaner fish. Dis Aquat Organ 117**:** 121-131.

Gupta GS (2012). Pentraxins: The L-Type Lectins and the C-Reactive Protein as a Cardiovascular Risk. In Gupta, G.S. (Ed.) Animal Lectins: Form, Function and Clinical Applications. Vienna: Springer Vienna.

Gygi SP, Rochon Y, Franza BR & Aebersold R (1999). Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19**:** 1720-1730.

Haugland GT, Olsen A-B, Rønneseth A & Andersen L (2017). Lumpfish (*Cyclopterus lumpus* L.) develop amoebic gill disease (AGD) after experimental challenge with Paramoeba perurans and can transfer amoebae to Atlantic salmon (*Salmo salar* L.). Aquaculture 478**:** 48-55.

Helland S, Dahle S, Hough C & Borthen J (2014). Production of ballan wrasse (*Labrus bergylta*). *Science and Practice The Norwegian Seafood Research Fund (FHF).*

Hjeltnes B B, G, Jansen, M D, Haukaas, a, & Walde (2017). The health situation in Norwegian aquaculture 2016. National Veterinary Institute 2017.

Huang ZH, Ma AJ & Wang XA (2011). The immune response of turbot, Scophthalmus maximus (L.), skin to high water temperature. J Fish Dis 34**:** 619-627. DOI 10.1111/j.1365-2761.2011.01275.x

Imsland AK, Reynolds P, Eliassen G, Hangstad TA, Foss A, Vikingstad E, et al. (2014a). The use of lumpfish (Cyclopterus lumpus L.) to control sea lice (Lepeophtheirus salmonis Krøyer) infestations in intensively farmed Atlantic salmon (Salmo salar L.). Aquaculture 424-425**:** 18-23.

Imsland AK, Reynolds P, Eliassen G, Hangstad TA, Nytrø AV, Foss A, et al. (2014b). Assessment of growth and sea lice infection levels in Atlantic salmon stocked in small-scale cages with lumpfish. Aquaculture 433**:** 137-142.

Imsland AK, Reynolds P, Eliassen G, Hangstad TA, Nytrø AV, Foss A, et al. (2015a). Assessment of suitable substrates for lumpfish in sea pens. Aquacult Int 23**:** 639-645. DOI 10.1007/s10499-014-9840-0

Imsland AK, Reynolds P, Eliassen G, Hangstad TA, Nytrø AV, Foss A, et al. (2015b). Feeding preferences of lumpfish (Cyclopterus lumpus L.) maintained in open net-pens with Atlantic salmon (Salmo salar L.). Aquaculture 436**:** 47-51.

Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. (2005). Exponentially Modified Protein Abundance Index (emPAI) for Estimation of Absolute Protein Amount in Proteomics by the Number of Sequenced Peptides per Protein. Mol Cell Proteom 4**:** 1265-1272.

Iversen A, Hermansen Ø, Andreassen O, Brandvik R, Marthinussen A & Nystøyl R (2015). Kostnadsdrivere i lakseoppdrett (Cost Drivers in Salmon Farming) [in Norwegian]. Nofima

Iversen MH & Eliassen RA (2014). The effect of allostatic load on hypothalamic–pituitary–interrenal (HPI) axis before and after secondary vaccination in Atlantic salmon postsmolts (Salmo salar L.). Fish Physiol Biochem 40**:** 527-538. DOI 10.1007/s10695-013-9863-x

Iwama GK, Afonso LOB, Todgham A, Ackerman P & Nakano K (2004). Are hsps suitable for indicating stressed states in fish? J Exp Biol 207**:** 15.

Jensen LB, Boltana S, Obach A, Mcgurk C, Waagbø R & Mackenzie S (2015a). Investigating the underlying mechanisms of temperature-related skin diseases in Atlantic salmon, Salmo salar L., as measured by quantitative histology, skin transcriptomics and composition. J Fish Dis 38**:** 977-992. DOI 10.1111/jfd.12314

Jensen LB, Wahli T, Mcgurk C, Eriksen TB, Obach A, Waagbø R, et al. (2015b). Effect of temperature and diet on wound healing in Atlantic salmon (Salmo salar L.). Fish Physiol Biochem 41**:** 1527-1543. DOI 10.1007/s10695-015-0105-2

Jurado J, Fuentes-Almagro CA, Guardiola FA, Cuesta A, Esteban MÁ & Prieto-Álamo MJ (2015). Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*). J Proteomics 120**:** 21-34.

Kamiya H, Muramoto K & Goto R (1988). Purification and properties of agglutinins from conger eel, Conger myriaster (Brevoort), skin mucus. Dev Comp Immunol 12**:** 309-318.

Kaplan A, Bueno M & Fournier AE (2017). Extracellular functions of 14-3-3 adaptor proteins. Cell Signal 31**:** 26-30.

Karlsen C, Thorarinsson R, Wallace C, Salonius K & Midtlyng PJ (2017). Atlantic salmon winter-ulcer disease: Combining mortality and skin ulcer development as clinical efficacy criteria against Moritella viscosa infection. Aquaculture 473**:** 538-544.

Kelata K (2009). Morphological analysis of chromatophores in the skin of trout. Bull Veterinary Institute Puławy 53**:** 117-121.

Kennedy J, Fréchet, A., Durif, C., Ólafsson, H. G. & Hedeholm, R. (2015b). Lumpfish Working Group Report. Reykjavik: Marine Research Institute. .

Kikuchi K, Watabe S & Aida K (1997). The Wap65 gene expression of goldfish (*Carassius auratus*) in association with warm water temperature as well as bacterial lipopolysaccharide (LPS). Fish Physiol Biochem 17**:** 423-432. DOI 10.1023/A:1007768531655

Kodama M, Sato S, Ogata T, Suzuki Y, Kaneko T & Aida K (1986). Tetrodotoxin secreting glands in the skin of puffer fishes. Toxicon 24**:** 819-829.

Kültz D, Chakravarty D & Adilakshmi T (2001). A novel 14-3-3 gene is osmoregulated in gill epithelium of the euryhaline teleost *Fundulus heteroclitus*. J Exp Biol 204**:** 2975-2985.

Kumar Mittal A & Whitear M (1979). Keratinization of fish skin with special reference to the catfish Bagarius bagarius. Cell Tissue Res 202**:** 213-230. DOI 10.1007/BF00232236

Le Guellec DM-D, Ghislaine. And Sire, Jean-Yves (2004). Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (*Danio rerio*). Int J Dev Biol 48**:** 217-231.

Le Morvan C, Troutaud D & Deschaux P (1998). Differential effects of temperature on specific and nonspecific immune defences in fish. J Exp Biol 201**:** 165.

Liu B-L, Jia R, Huang B & Lei J-L (2017). Interactive effect of ammonia and crowding stress on ion-regulation and expression of immune-related genes in juvenile turbot (Scophthalmus maximus). Mar Freshw Behav Physiol 50**:** 179-194. DOI 10.1080/10236244.2017.1360726

Liu S, Wang X, Sun F, Zhang J, Feng J, Liu H, et al. (2013). RNA-Seq reveals expression signatures of genes involved in oxygen transport, protein synthesis, folding, and degradation in response to heat stress in catfish. Physiol Genom 45**:** 462-476. DOI 10.1152/physiolgenomics.00026.2013

Liu Y & Bjelland HV (2014). Estimating costs of sea lice control strategy in Norway. Prev Vet Med 117**:** 469-477.

Lopez MF, Berggren K, Chernokalskaya E, Lazarev A, Robinson M & Patton WF (2000). A comparison of silver stain and SYPRO Ruby Protein Gel Stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. Electrophoresis 21**:** 3673-3683. DOI 10.1002/1522-2683(200011)21:17<3673::AID-ELPS3673>3.0.CO;2-M

Magnadóttir B (2006). Innate immunity of fish (overview). Fish Shellfish Immunol 20**:** 137-151.

Maier T, Güell M & Serrano L (2009). Correlation of mRNA and protein in complex biological samples. FEBS Lett 583**:** 3966-3973.

Malo MS, Alam SN, Mostafa G, Zeller SJ, Johnson PV, Mohammad N, et al. (2010). Intestinal alkaline phosphatase preserves the normal homeostasis of gut microbiota. Gut 59**:** 1476.

Marcos-López M, Donald K, Stagg H & Mccarthy Ú (2013). Clinical *Vibrio anguillarum* infection in lumpsucker *Cyclopterus lumpus* in Scotland. Vet Rec 173**:** 319.

Mateus AP, Anjos L, Cardoso JR & Power DM (2017). Chronic stress impairs the local immune response during cutaneous repair in gilthead sea bream (Sparus aurata, L.). Mol Immunol 87**:** 267-283.

Mcewen BS (2000). Allostasis and Allostatic Load: Implications for Neuropsychopharmacology. Neuropsychopharmacol 22: 108. DOI 10.1016/S0893-133X(99)00129-3

Mcewen BS (2005). Stressed or stressed out: What is the difference? J Psychiat Neurosci 30: 315-318.

Mcewen BS & Wingfield JC (2003). The concept of allostasis in biology and biomedicine. Horm Behav 43: 2-15.

Mecklenburg C & Sheiko BA (2003). Family Cyclopteridae Bonaparte 1831 lumpsuckers. Calif Acad Sci Annot Checkl Fish.

Mitamura H, Thorstad EB, Uglem I, Bjørn PA, Økland F, Næsje TF, et al. (2012). Movements of lumpsucker females in a northern Norwegian fjord during the spawning season. Environ Biol Fish 93: 475-481. DOI 10.1007/s10641-011-9942-8

Mittal AK, Ueda T, Fujimori O & Yamada K (1994). Histochemical analysis of glycoproteins in the unicellular glands in the epidermis of an Indian freshwater fish *Mastacembelus pancalus* (Hamilton). Histochem J 26: 666-677.

Molle V, Campagna S, Bessin Y, Ebran N, Saint N & Molle G (2008). First evidence of the pore-forming properties of a keratin from skin mucus of rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri). Biochem J 411: 33.

Moring JR (1994). Color Phases of Lumpfish Fry. Mar Natural 2: 11-14. DOI 10.2307/3858154

Nytrø AV, Vikingstad E, Foss A, Hangstad TA, Reynolds P, Eliassen G, et al. (2014). The effect of temperature and fish size on growth of juvenile lumpfish (Cyclopterus lumpus L.). Aquaculture 434: 296-302.

Øines Ø & Heuch PA (2007). Caligus elongatus Nordmann genotypes on wild and farmed fish. J Fish Dis 30: 81-91. DOI 10.1111/j.1365-2761.2007.00783.x

Ottesen OH & Olafsen JA (1997). Ontogenetic development and composition of the mucous cells and the occurrence of saccular cells in the epidermis of Atlantic halibut. J Fish Biol 50: 620-633.

Paffenholz R & Franke WW (1997). Identification and localization of a neurally expressed member of the plakoglobin/armadillo multigene family. Differentiation 61: 293-304.

Palaksha KJ, Shin G-W, Kim Y-R & Jung T-S (2008). Evaluation of non-specific immune components from the skin mucus of olive flounder (Paralichthys olivaceus). Fish Shellfish Immunol 24: 479-488.

Parseghian MH & Luhrs KA (2006). Beyond the walls of the nucleus: the role of histones in cellular signaling and innate immunity. Biochem Cell Biol 84: 589-595. DOI 10.1139/o06-082

Patel DM & Brinchmann MF (2017). Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). Biochem Biophys Rep 9: 217-225.

Patterson LB & Parichy DM (2013). Interactions with Iridophores and the Tissue Environment Required for Patterning Melanophores and Xanthophores during Zebrafish Adult Pigment Stripe Formation. PLOS Genetics 9: e1003561. DOI 10.1371/journal.pgen.1003561

Patwari P, Higgins LJ, Chutkow WA, Yoshioka J & Lee RT (2006). The Interaction of Thioredoxin with Txnip: Evidence for Formation of a Mixed Disulfide by Disulfide Exchange. J Biologic Chem 281**:** 21884-21891. DOI 10.1074/jbc.M600427200

Pfeiffer W (1977). The Distribution of Fright Reaction and Alarm Substance Cells in Fishes. Copeia 1977**:** 653-665. DOI 10.2307/1443164

Pickering AD & Fletcher JM (1987). Sacciform cells in the epidermis of the brown trout, Salmo trutta, and the Arctic char, Salvetinus alpinus. Cell Tissue Res 247**:** 259-265. DOI 10.1007/BF00218307

Pittman K, Pittman A, Karlson S, Cieplinska T, Sourd P, Redmond K, et al. (2013). Body site matters: an evaluation and application of a novel histological methodology on the quantification of mucous cells in the skin of Atlantic salmon, Salmo salar L. J Fish Dis 36**:** 115-127. DOI 10.1111/jfd.12002

Powell A, Treasurer JW, Pooley CL, Keay AJ, Lloyd R, Imsland AK, et al. (2017). Use of lumpfish for sea-lice control in salmon farming: challenges and opportunities. Rev Aquacult**:** n/a-n/a. DOI 10.1111/raq.12194

Pridgeon JW & Klesius PH (2013). Apolipoprotein A1 in channel catfish: Transcriptional analysis, antimicrobial activity, and efficacy as plasmid DNA immunostimulant against *Aeromonas hydrophila* infection. Fish Shellfish Immunol 35**:** 1129-1137.

Quiniou SMA, Bigler S, Clem LW & Bly JE (1998). Effects of water temperature on mucous cell distribution in channel catfish epidermis: a factor in winter saprolegniasis. Fish Shellfish Immunol 8**:** 1-11.

Rai AK & Mittal AK (1983). Histochemical response of alkaline phosphatase activity during the healing of cutaneous wounds in a cat-fish. Experientia 39**:** 520-522. DOI 10.1007/BF01965185

Rajan B, Fernandes JMO, Caipang CMA, Kiron V, Rombout JHWM & Brinchmann MF (2011). Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. Fish Shellfish Immunol 31**:** 224-231.

Rajan B, Kiron V, Fernandes JMO & Brinchmann MF (2013a). Localization and functional properties of two galectin-1 proteins in Atlantic cod (*Gadus morhua*) mucosal tissues. Dev Comp Immunol 40**:** 83-93.

Rajan B, Lokesh J, Kiron V & Brinchmann MF (2013b). Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*. BMC Vet Res 9**:** 103.

Rajan B, Patel DM, Kitani Y, Viswanath K & Brinchmann MF (2017a). Novel mannose binding natterin-like protein in the skin mucus of Atlantic cod (*Gadus morhua*). Fish Shellfish Immunol 68**:** 452-457.

Rajan B, Patel DM, Kitani Y, Viswanath K & Brinchmann MF (2017b). Novel mannose binding natterin-like protein in the skin mucus of Atlantic cod (Gadus morhua). Fish Shellfish Immunol 68**:** 452-457.

Rakers S, Gebert M, Uppalapati S, Meyer W, Maderson P, Sell AF, et al. (2010). 'Fish matters': the relevance of fish skin biology to investigative dermatology. Exp Dermatol 19**:** 313-324. DOI 10.1111/j.1600-0625.2009.01059.x

Rakers S, Niklasson L, Steinhagen D, Kruse C, Schauber J, Sundell K, et al. (2013). Antimicrobial peptides (AMPs) from fish epidermis: Perspectives for investigative dermatology. J Invest Dermatol 133**:** 1140-1149. DOI 10.1038/jid.2012.503

Ralphs JR & Benjamin M (1992). Chondroitin and keratan sulphate in the epidermal club cells of teleosts. J Fish Biol 40**:** 473-475. DOI 10.1111/j.1095-8649.1992.tb02594.x

Ramsay DS & Woods SC (2014). Clarifying the Roles of Homeostasis and Allostasis in Physiological Regulation. Psychol Rev 121**:** 225-247. DOI 10.1037/a0035942

Rebl A, Goldammer T & Seyfert H-M (2010). Toll-like receptor signaling in bony fish. Vet Immunol Immunop 134**:** 139-150.

Reddy VM, Suleman FG & Hayworth DA (2004). Mycobacterium avium binds to mouse intestinal mucus aldolase. Tuberculosis 84**:** 303-310.

Rogowska-Wrzesinska A, Le Bihan M-C, Thaysen-Andersen M & Roepstorff P (2013). 2D gels still have a niche in proteomics. J Proteomics 88**:** 4-13.

Rønneseth A, Ghebretnsae DB, Wergeland HI & Haugland GT (2015). Functional characterization of IgM+ B cells and adaptive immunity in lumpfish (Cyclopterus lumpus L.). Dev Comp Immunol 52**:** 132-143.

Salinas I (2015). The Mucosal Immune System of Teleost Fish. Biology 4**:** 525-539. DOI 10.3390/biology4030525

Saurabh S & Sahoo PK (2008). Lysozyme: an important defence molecule of fish innate immune system. Aquac Res 39**:** 223-239. DOI 10.1111/j.1365-2109.2007.01883.x

Sayer MDJ & Davenport J (1996). Hypometabolism in torpid goldsinny wrasse subjected to rapid reductions in seawater temperature. J Fish Biol 49**:** 64-75. DOI 10.1111/j.1095-8649.1996.tb00005.x

Sayer MDJ & Reader JP (1996). Exposure of goldsinny, rock cook and corkwing wrasse to low temperature and low salinity: survival, blood physiology and seasonal variation. J Fish Biol 49**:** 41-63. DOI 10.1111/j.1095-8649.1996.tb00004.x

Selye H (1973). The Evolution of the Stress Concept: The originator of the concept traces its development from the discovery in 1936 of the alarm reaction to modern therapeutic applications of syntoxic and catatoxic hormones. Am Sci 61**:** 692-699.

Sha Z, Xu P, Takano T, Liu H, Terhune J & Liu Z (2008). The warm temperature acclimation protein Wap65 as an immune response gene: Its duplicates are differentially regulated by temperature and bacterial infections. Mol Immunol 45**:** 1458-1469.

Shen Y, Zhang J, Xu X, Fu J & Li J (2012). Expression of complement component C7 and involvement in innate immune responses to bacteria in grass carp. Fish Shellfish Immunol 33**:** 448-454.

Skiftesvik AB, Bjelland RM, Durif CMF, Johansen IS & Browman HI (2013). Delousing of Atlantic salmon (Salmo salar) by cultured vs. wild ballan wrasse (Labrus bergylta). Aquaculture 402-403**:** 113-118.

Sköld HN, Amundsen T, Svensson PA, Mayer I, Bjelvenmark J & Forsgren E (2008). Hormonal regulation of female nuptial coloration in a fish. Horm Behav 54**:** 549-556.

Småge SB, Frisch K, Brevik ØJ, Watanabe K & Nylund A (2016). First isolation, identification and characterisation of Tenacibaculum maritimum in Norway, isolated from diseased farmed sea lice cleaner fish Cyclopterus lumpus L. Aquaculture 464**:** 178-184.

Stafford JL & Belosevic M (2003). Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Dev Comp Immunol 27**:** 539-554.

Sun P, Bao P & Tang B (2017). Transcriptome analysis and discovery of genes involved in immune pathways in large yellow croaker (Larimichthys crocea) under high stocking density stress. Fish Shellfish Immunol 68**:** 332-340.

Tacchi L, Musharrafieh R, Larragoite ET, Crossey K, Erhardt EB, Martin SaM, et al. (2014). Nasal immunity is an ancient arm of the mucosal immune system of vertebrates. Nat Commun 5**:** 5205-5205. DOI 10.1038/ncomms6205

Tian Q, Stepaniants SB, Mao M, Weng L, Feetham MC, Doyle MJ, et al. (2004). Integrated Genomic and Proteomic Analyses of Gene Expression in Mammalian Cells. Mol Cell Proteom 3**:** 960-969.

Tomanek L & Sanford E (2003). Heat-Shock Protein 70 (Hsp70) as a Biochemical Stress Indicator: an Experimental Field Test in Two Congeneric Intertidal Gastropods (Genus: Tegula). Biologic Bull 205**:** 276-284. DOI 10.2307/1543291

Tort L (2011). Stress and immune modulation in fish. Dev Comp Immunol 35**:** 1366-1375.

Treasurer JW (2002). A review of potential pathogens of sea lice and the application of cleaner fish in biological control. Pest Manag Sci 58**:** 546-558. DOI 10.1002/ps.509

Tripathi P & Mittal AK (2010). Essence of keratin in lips and associated structures of a freshwater fish Puntius sophore in relation to its feeding ecology: Histochemistry and scanning electron microscope investigation. Tissue Cell 42**:** 223-233.

Ulvila J, Vanha-Aho LM, Kleino A, Vähä-Mäkilä M, Vuoksio M, Eskelinen S, et al. (2011). Cofilin regulator 14-3-3ζ is an evolutionarily conserved protein required for phagocytosis and microbial resistance. J Leukocyte Biol 89**:** 649-659. DOI 10.1189/jlb.0410195

Vasconcelos P, Monteiro CC, Santos MN & Gaspar MB (2004). First record of the lumpfish (Cyclopterus lumpus Linnaeus, 1758) off the Algarve coast (southern Portugal): southward extension of the species distributional range. J Appl Ichthyol 20**:** 159-160. DOI 10.1046/j.1439-0426.2003.00531.x

Vatsos IN, Kotzamanis Y, Henry M, Angelidis P & Alexis MN (2010). Monitoring stress in fish by applying image analysis to their skin mucous cells. Eur J Histochem 54**:** e22. DOI 10.4081/ejh.2010.e22

Verner-Jeffreys DW, Pond MJ, Peeler EJ, Rimmer GSE, Oidtmann B, Way K, et al. (2008). Emergence of cold water strawberry disease of rainbow trout Oncorynchus mykiss in England and Wales: outbreak investigations and transmission studies. Dis Aquat Organ 79**:** 207-218.

Wakabayashi H (1991). Effect of environmental conditions on the infectivity of Flexibacter columnaris to fish. J Fish Dis 14**:** 279-290. DOI 10.1111/j.1365-2761.1991.tb00825.x

Wang Z, Gerstein M & Snyder M (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10**:** 57. DOI 10.1038/nrg2484

Wein S, Fauroux M, Laffitte J, De Nadaï P, GuaïNi C, Pons F, et al. (2004). Mediation of annexin 1 secretion by a probenecid-sensitive ABC-transporter in rat inflamed mucosa. Biochem Pharmacol 67**:** 1195-1202.

Wendelaar Bonga SE (1997). The stress response in fish. Physiol Rev 77**:** 591-625. DOI 10.1152/physrev.1997.77.3.591

Wendelaar Bonga SE (2011). Hormonal responses to stress. In Anthony, P. (Ed.) Encyclopedia of Fish Physiology San Diego: Academic Press.

Wingfield JC (2005). The Concept of Allostasis: Coping With a Capricious Environment. J Mammal 86**:** 248-254. DOI 10.1644/BHE-004.1

Xu Z, Parra D, Gómez D, Salinas I, Zhang Y-A, Von Gersdorff Jørgensen L, et al. (2013). Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. P Natl Acad Sci USA 110**:** 13097-13102.

Yamamoto M, Okamoto T, Takeda K, Sato S, Sanjo H, Uematsu S, et al. (2006). Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling. Nat Immunol 7**:** 962. DOI 10.1038/ni1367

Zhao F, Li Y-W, Pan H-J, Shi C-B, Luo X-C, Li A-X, et al. (2013). Expression profiles of toll-like receptors in channel catfish (Ictalurus punctatus) after infection with Ichthyophthirius multifiliis. Fish Shellfish Immunol 35**:** 993-997.

Paper I

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Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*)

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ABSTRACT

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, NCBInr and vertebrate EST. The identified proteins were clustered into ten groups based on their gene ontology biological process in PANTHER (www.patherdb.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 lumsucker'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 (*Lepeoptheirus 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,

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further held at Mørkvedbukta Research Station, Bodø, 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 to 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, USA). Next, the sonicated skin mucus was centrifuged at 15,000*g* 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 10000*g* 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 10,000*g* 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% Biolyte (3–10), and 0.001% bromophenol blue, all from Sigma, except Biolyte 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), 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 iso-electric focusing in Bio-Rad Protean IEF cell to a total volt hours of 60,000 at a maximum of 10,000 V using three steps of slow ramping at a constant temperature of 20 °C [5]. The focused IPG strips were reduced with 0.2% DTT and alkylated with 0.3% iodoacetamide for 15 min each in equilibration buffer (6 M urea, VWR; 0.375 M tris-HCl (pH 8.8), Bio-Rad; 2% SDS, 20% glycerol, Sigma). The equilibrated gel strips were loaded on 12.5% polyacrylamide gels in the Bio-Rad Protean IIxii system (USA). Initially, the gels were run at constant current of 20 mA/gel for 15 min and then 6 mA/gel overnight (approx. 16 h). The following day, current was increased to 15 mA/gel to complete the run. The voltage was limited to 250 V throughout the run. The gels were stained with Sypro® Ruby Protein gel stain, Life technologies, USA, following the manufacturers protocol and images were documented using ChemiDoc™ XRS system (Bio-Rad). The documented gel images were analyzed in PDQuest™ Advanced 2D analysis software (Bio-Rad) to identify consistent spots over 6 gels. Fifty spots with high expression levels in the skin mucus of lumpsucker were selected for analysis.

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 pkl (powered keylogger) files by using the Protein Lynx Global server software (version 2.1, Micromass/Waters, MA, USA). The pkl 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, 69146588 sequences). In places where SwissProt or NCBInr 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 retrived 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 pI 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

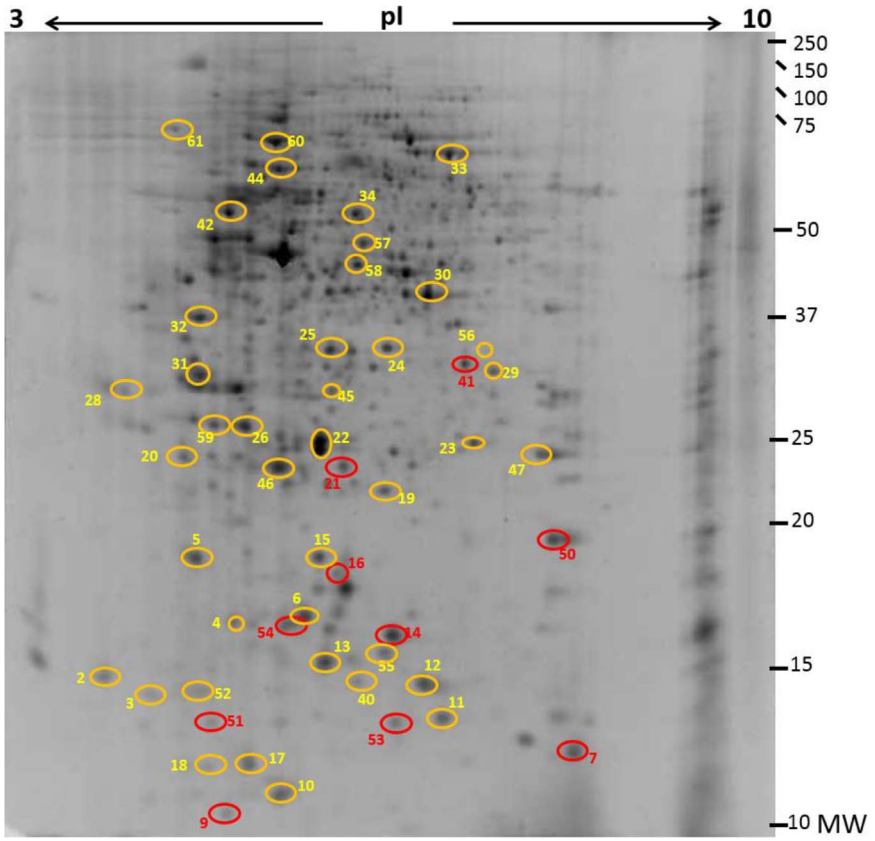

Fig. 1. *Cyclopterus lumpus* skin mucus was sonicated, acid precipitated and dissolved in rehydration buffer (Section 2.2) then 80 μg were loaded unto 17 cm, 3–10 non-linear IPG strips. 12.5% polyacrylamide vertical gels were used as the second dimension. The image shows a representative gel with the spots analyzed with mass spectrometry circled. Yellow rings represent identified spots, red rings; not identified spots.

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 thioredoxin 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 ovatus*) 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 important 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 production 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 notothenioid 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 lumpsucker skin mucus.

Spot ID	Protein name (Species)	Gene symbol [a]	Accession number	Observed PI/MW	Apparent PI/MW	ST[b]	Protein score/Total score of Up[c]	SC[d] (%)	Peptide sequence[e]
L2	Calmodulin (*Ctenopharyngodon idella*)	*calm*	Q6IT78	15/3.7	16.8/4.09	28	84/84	32	**KELGTVMRS** **KDTDSEEEIRE**
L3	Histone H2B 1/2 (*Danio rerio*)	*hist1h2b*	Q5BJA5	14.3/4.1	13.5/10.37	19	40/40	5	**REIQTAVRL**
L4	Predicted: Lipocalin-like (*Xiphophorus maculates*)	*lcn1*	XP_005803374	17.9/5	21.3/4.94	49	57/57	4	**KDGVSEVLNKL**
L5	Myosin, light polypeptide 9, like 1 (*Salmo salar*)	*myl9*	ACH70953	21.3/4.5	19.8/4.69	57	130/67	29	KEAFNMIDQNRD **RFTDEEVDELFRE**
L6	Growth/differentiation factor 6-A (*D. rerio*)	*gdf6a*	P85857	18.2/5.6	46.8/9.21	20	22/22	3	**KFLKEILASSPGASRR**
L10	Glial fibrillary acidic protein (*Carassius auratus*)	*gfap*	P48677	10.8/5.4	42.6/4.93	19	51/51	3	**KLALDIEIATYRK**
L11	Predicted: Cystatin-B-like (*Oryzias latipes*)	*cstb*	XP_004081114	13.5/7	11.1/6.49	53	85/85	12	**KTQVVAGTNYFIKV**
L12	Nucleoside diphosphate kinase B (*Macruronus magellanicus*)	*nme2*	P85292	14.9/6.8	14.2/5.70	22	62/62	9	**TFIAIKPDGVQRG**
L13	Histone H3.2 (*D. rerio*)	*hist1h3*	Q4QRF4; A2VD42	15.9/5.8	15.4/11.27	20	25/25	5	**REIAQDFKT**
L15	60S ribosomal protein L11 (*Ictalurus punctatus*)	*rpl11*	Q90YV7	21.6/5.8	20.6/10.07	33	37/37	3	**KAEEILEKG**
L17	Glial fibrillary acidic protein (*C. auratus*)	*gfap*	P48677	11.9/5.1	42.6/ 4.93	24	92/65	4	RFLEQQNKM **KLALDIEIATYRK**
L18	Glial fibrillary acidic protein (*C. auratus*)	*gfap*	P48677	11.6/4.6	42.6/4.93	23	56/56	4	**KLALDIEIATYRK**
L19	Peroxiredoxin 1 (*Oryzias melastigma*)	*prdx1*	AEA51065	26.1/6.4	22/6.30	57	266/129	29	RGLFVIDDKG **KEDDGIAYRG** **KIPLVADLTKS** RQITINDLPVGRS
L20	Predicted: apolipoprotein A-I-like (*Gasterosteus aculates*)	*apoa1*	CD493099	28.4/4.4	26.7/4.69	59	70/70	9	**KEIAAPYVTNLKG**
L22	Natterin-2 (*Thalassophryne nattereri*)	N/A	Q66S21	29.9/5.8	41.9/8.90	24	34/34	2	**KADIPFTATLIRT**
L23	Triosephosphate isomerase B (*D. rerio*)	*tpI1*	Q90XG0; Q7T315;	29.9/7.8	27/6.45	23	110/59	15	**KGAFTGEISPAMIKD** RHVFGESDELIGQKV
L24	Keratin, type I cytoskeletal 13 (*Oncorhynchus mykiss*)	*krt13*	Q8JFQ6	39.4/6.4	51.9/5.17	19	56/56	1	**KLAADDFRT**
L25	Predicted: F-actin-capping protein subunit beta isoforms 1 and 2-like isoform X1 (*Oreochromis niloticus*)	*capzb*	XP_003441481	39.3/5.8	30.8/5.82	53	127/127	26	**RSTLNEIYFGKT** **KTGSGTMNLGGSLTRQ**
L26	14-3-3 protein beta/alpha-A (*D. rerio*)	*ywhab*	Q5PRD0; A3KNI9	31.4/5	27.7/4.71	24	259/54	18	RVISSIEQKT RNLLSVAYKN **RYDDMAASMKA** RYLSEVASGDSKR KDSTLIMQLLRD
L28	14-3-3 protein beta/alpha-1 (*O. mykiss*)	*ywhab*	Q6UFZ9	34.1/3.7	27.6/4.64	20	78/78	7	**RNLLSVAYKN** **KDSTLIMQLLRD**
L29	Guanine nucleotide-binding protein subunit beta-2-like 1 (*D. rerio*)	*gnb2l1*	O42248	36.7/8.2	35.5/7.60	30	93/93	5	**KIIVDELRQ** **RDETNYGIPQRA**
L30	Glyceraldehyde 3-phosphate dehydrogenase isoform 2 (*Oplegnathus fasciatus*)	*gapdh*	ACF35053	45.7/6.8	36.3/6.20	43	87/87	9	**KYVVESTGVFLSVEKA**
L31	Charged multivesicular body protein 4c (*D. rerio*)	*chmp4c*	Q6IQ73; Q803U4	36/4.6	25.1/4.71	25	112/112	9	**RETEEMLAKK** **REALENANTNTEVLKN**
L32	14-3-3 protein beta/alpha-B (*D. rerio*)	*ywhab*	Q7T356	42.7/4.5	27.4/4.68	24	164/46	16	**RYDDMAAAMKA** KDSTLIMQLLRD KAVTEGGVELSNEERN
L33	Keratin, type I cytoskeletal 13 (*O. mykiss*)	*krt13*	Q8JFQ6	68.1/7	51.9/5.17	24	31/31	1	**KLAADDFRT**
L34	Alpha-enolase (*Thunnus albacores*)	*eno1*	I0J1J1; B3A0L7	57.8/6.1	47.5/7	26	639/586	28	**RGNPTVEVDLYTKK** KFGANAILGVSLAVCKA **KIVIGMDVAASEFYKG** **KIDKLMLDMDGTENKY** **RAAVPSGASTGIYEALELRD** **KLAMQEFMILPVGASSFKD** **KFTASTSIQVVGDDLTVTNPKR**
L40	Nucleoside diphosphate kinase B (*M. magellanicus*)	*nme2*	P85292	14.9/6.2	14.2/5.70	20	33/33	9	**TFIAIKPDGVQRG**

(continued on next page)

Table 1 *(continued)*

L42	ATP synthase subunit beta, mitochondrial (*Cyprinus carpio*)	ATP5b	Q9PTY0	58.2/4.8	55.3/5.05	30	876/876	37	**KVLDTGAPIRI** **KIGLFGGAGVGKT** **RIPVGPETLGRI** **RTIAMDGTEGLVRG** **RIMNVIGEPIDERG** **RFTQAGSEVSALLGRI** **KTVLIMELINNVAKA** **KVALVYGQMNEPPGARA** **RDQEGQDVLLFIDNIFRF** **RAIAELGIYPAVDPLDSTSRI** **REGNDLYHEMIESGVINLKD** **RIPSAVGYQPTLATDMGTMQERI**
L44	Warm-temperature-acclimation-related 65 kDa protein (*O. fasciatus*)	*wap65*	AFE88226	66.1/5.3	49.2/5.36	48	66/66	7	**KEIQEDFPGVPSHLDAAVECPKG**
L45	Predicted: actin, cytoplasmic-like (*Pundamilia nyererei*)	*actb*	XP_005754844	34.8/5.9	28.9/5.20	48	209/81	27	RGYSFTTTAERE **KQEYDESGPAIVHRK** KSYELPDGQVITIGNERF
L46	Predicted: pentraxin fusion protein-like (*Cynoglossus semilaevis*)	*pxn*	XP_008323044	28.1/5.3	25.4/5.43	45	46/46	7	**RVATELKGERE**
L47	Glutathione S-transferase Mu-3 like (*Dicentrarchus labrax*)	*gstm3*	AM984133	29/6.7	22138/6.49	56	135/72	26	KIVQSNAIMRY **KLGMDLPNLPYLEDGDRKI**
L52	Coactosin-like protein (*Notothenia coriiceps*)	*cotl1*	XP_010785783	14.5/4.6	10.3/6.26	46	101/101	46	**RELDADNIRS** **KFTLITWIGENISGLQRA**
L55	DNA-binding protein RFX2 (*D. rerio*)	*rfx2*	Q5EAP5	16.3/6.4	82.2/6.36	19	24/24	1	**KLITLCKYEPIKQ**
L56	Malate dehydrogenase 2-2, NAD (mitochondrial) (*S. salar*)	*mdh2*	NP_001133198	39/8.1	35.7/8.15	55	484/81	33	RIQDAGTEVVKA RVFGVTTLDIVRA RFTFSVLDAMNGKE KAGAGSATLSMAYAGARF KVAVLGASGGIGQPLSLLLKN **RDDLFNTNATIVATLADAVARN**
L57	Actin cytoplasmic 1 (*C. idella*)	*actb*	P83751; O73815; P12714	53.2/6.1	42.0/5.30	20	65/65	12	**RDLTDYLMKI** **RGYSFTTTAERE**
L58	Predicted: septin-2 (*C. semilaevis*)	*sept2*	XP_008334373	50.1/6.1	40.2/5.94	50	156/156	10	**RILDEIAEQGIRI** **KTIIQYIDNQFERY**
L59	14-3-3 protein beta/alpha-1-like (*X. maculatus*)	*ywhab*	XP_005805709	31.6/4.7	27.6/4.62	43	162/113	17	**RYDDMAAAMKS** RYLSEVASGDSKK **KSVTEQGAELSNEERN**
L60	Heat shock 70 kDa protein 8b1 (*Monopterus albus*)	*hspa8*	AGO01988	71/5.3	70.9/5.44	55	1234/69	37	KDAGTISGLNVLRI KVEIIANDQGNRT RFEELNADLFRG RMVNHFISEFKR KMKEIAEAYLGKT KNGLESYAFNMKS RTTPSYVAFTDTERL KNQVALNPNNTVFDAKR RIINEPTAAAIAYGLDKK **KTFYPEEISSMVLIKM** KSTAGDTHLGGEDFDNRM RIINEPTAAAIAYGLDKKV KSINPDEAVAYGAAVQAAILSGDKS KQTQTFTTYSDNQPGVLIQVFEGERA
L61	Heat shock cognate 71 kDa (*D. rerio*)	*hspa8*	Q90473	73/4.2	71.1/5.18	19	97/97	4	**KVEIIANDQGNRT** **RTTPSYVAFTDTERL**

a) Gene symbol retrived from UniProtKB
b) Significant threshold score
c) Total score of unique peptides
d) Sequence coverage
e) Unique peptide sequences are in red

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 lumpsucker 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].

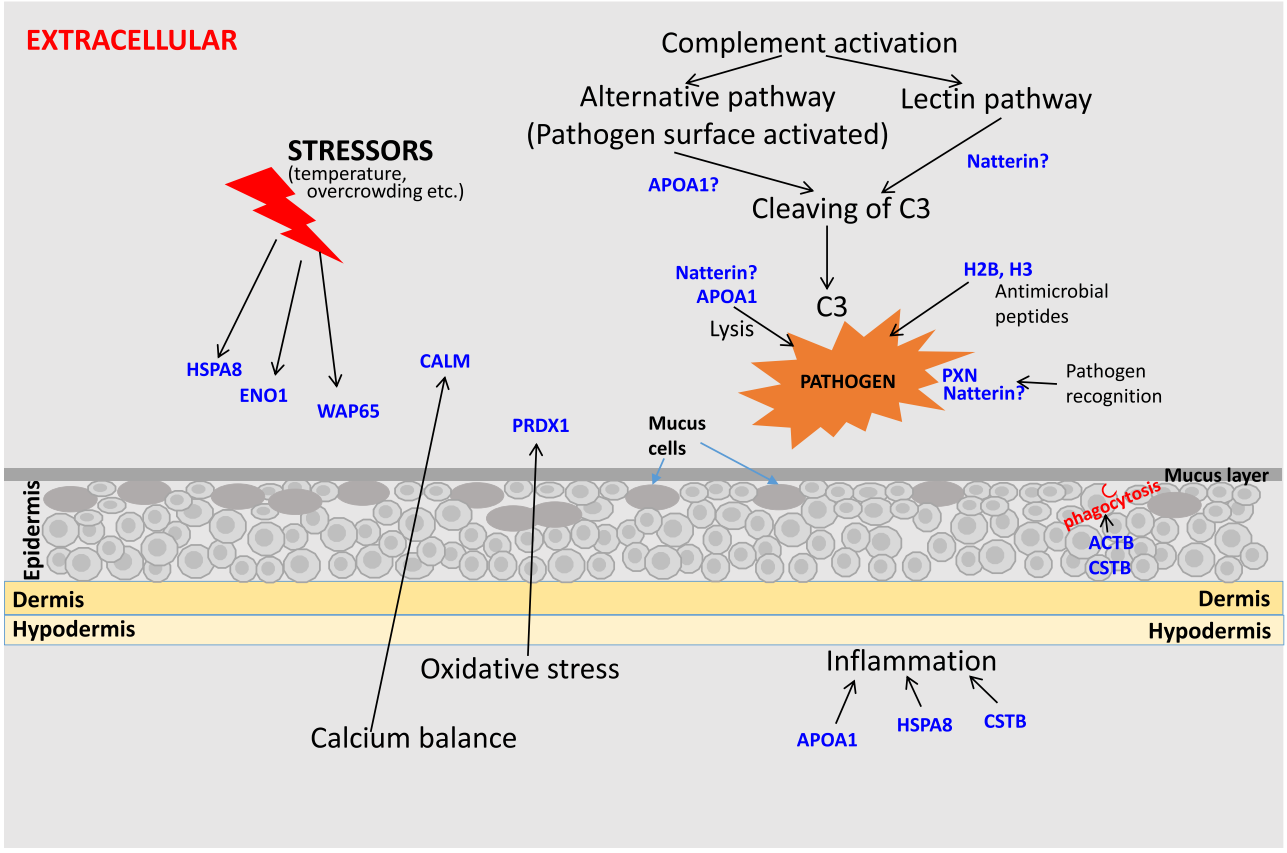

Fig. 2. Possible interactions of some of the identified proteins from lumpsucker skin mucus are shown. Proteins in the figure are indicated by their abbreviations. Arrows indicates their involvement in different process. Question mark "?" indicates proposed actions of the proteins. Biological roles of the proteins are explained in text in results and discussion section.

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 epidermis of Japanese eel (*Anguilla japonica)* has been found to inhibit the proteolytic activity of cysteine proteases of *Porphyromonas gingivalis* [40]. Significant changes in cystatin-B level was observed in Atlantic salmon infected by *Neoparamoeba perurans* [41], and in turbot (*Scopthalmus maximus)* infected by *Ectalurus tarda* [42]. In *S. maximus* cystatin-B were also involved in bacterial invasion of head kidney macrophages [42]. This protein has also been identified in skin mucus of Atlantic cod [5].

Apolipoprotein A1 (spot 20) is a major component of high-density lipoprotein in plasma mainly involved in lipid metabolism [43]. It also plays anti-inflammatory role in both acute and chronic inflammation [44]. This protein was upregulated in skin mucus of sea lice infected Atlantic salmon [11], *Vibrio anguillarum* infected Atlantic cod [6]. Furthermore, in channel catfish this protein also showed lytic activities against Gram positive *Micrococcus lysodeikticus* and Gram negative *Aeromonas hydrophila* [45]. The carboxyl end of this protein is responsible for antimicrobial activities that might give protection against pathogens in skin mucus of teleost fish (Fig. 2) [46]. Apolipoprotein A1 has also been identified 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 mirabilis* [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].

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 (*Gillicthys mirabilis*) [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 lumpsucker. These are highly conserved proteins found ubiquitously in animal tissues. They are signaling proteins associated

Table 2
GO biological process of identified proteins.

Spot ID	Protein name	Biological process										Reported in skin mucus	Present extracellularly
		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10		
L2	Calmodulin				✓							Y [6]	Y [55]
L3	Histone H2B 1/2			✓	✓				✓			Y	Y [56]
L4	Predicted: Lipocalin-like				✓			✓	✓			–	Y [57]
L5	Myosin, light polypeptide 9, like 1					✓				✓		–	–
L6	Growth/differentiation factor 6-A	✓	✓		✓	✓			✓		✓	–	Y
L10, L17,L18	Glial fibrillary acidic protein			✓	✓	✓						–	–
L11	Predicted: Cystatin-B-like		✓						✓			Y [5]	–
L12, L40	Nucleoside diphosphate kinase B	✓	✓		✓				✓			Y [5,8,9]	Y
L13	Histone H3.2			✓					✓			–	Y [56]
L15	60 S ribosomal protein L11								✓			Y [8]	Y
L19	Peroxiredoxin 1						✓		✓			Y [8]	Y [58]
L20	Predicted: apolipoprotein A-I-like		✓		✓	✓		✓	✓	✓	✓	Y [8,9]	Y
L22	Natterin-2											–	Y
L23	Triosephosphate isomerase B								✓			Y [5,8,9]	Y
L24, L33	Keratin, type I cytoskeletal 13			✓	✓	✓						Y [8]	Y
L25	Predicted: F-actin-capping protein subunit beta isoforms 1 and 2-like isoform X1		✓	✓	✓	✓			✓			–	–
L26, L28, L32, L59	14-3-3 protein beta/alpha				✓							Y [5,8]	Y [59]
L29	Guanine nucleotide-binding protein subunit beta-2-like 1				✓			✓				–	–
L30	Glyceraldehyde 3-phosphate dehydrogenase isoform 2								✓			Y [5,8,9]	–
L31	Charged multivesicular body protein 4c							✓				–	Y
L34	Alpha-enolase								✓			Y [5,8]	Y
L42	ATP synthase subunit beta, mitochondrial							✓	✓			Y [8,9]	Y
L44	Warm-temperature-acclimation-related 65 kDa protein							✓				Y [8,9]	Y
L45	Actin, cytoplasmic			✓	✓			✓				Y [5,9]	Y
L46	Predicted: pentraxin fusion protein-like						✓				✓	Y [27]	Y [27]
L47	Glutathione-S-transferase											Y [5,9]	Y
L52	Coactosin-like protein			✓	✓							Y [8,9]	Y
L55	DNA-binding protein RFX2		✓		✓				✓			–	–
L56	Malate dehydrogenase 2-2, NAD								✓			Y [9]	Y
L58	Predicted: septin-2				✓				✓			–	–
L60	Heat shock 70 kDa protein 8b1			✓			✓		✓		✓	Y [7,9]	Y [60]
L61	Heat shock cognate 71 kDa			✓			✓		✓		✓	Y [9]	Y [60]

B1; apoptotic process (GO:0006915), B2; biological regulation (GO:0065007), B3; cellular component organization or biogenesis (GO:0071840), B4; cellular process (GO:0009987), B5; developmental process (GO:0032502), B6; immune system process (GO:0002376), B7; locali
zation (GO:0051179), B8; metabolic process (GO:0008152), B9; multicellular organismal process (GO:0032501), B10; response to stimulus (GO:0050896).
"Y" means yes, the protein has been identified in skin mucus of fish or its extracellular presence has been observed. Information is based on UNiProtKB in places where references are not cited.

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 *Staphyloccocus 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:0002376), localization (GO:0051179), metabolic process (GO:0008152), multicellular organismal process (GO:0032501) and response to stimulus (GO:0050896). The GO biological process indicated the involvement of individual proteins in several processes, which are listed in Table 2. A confidence view (medium confidence score) 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, to

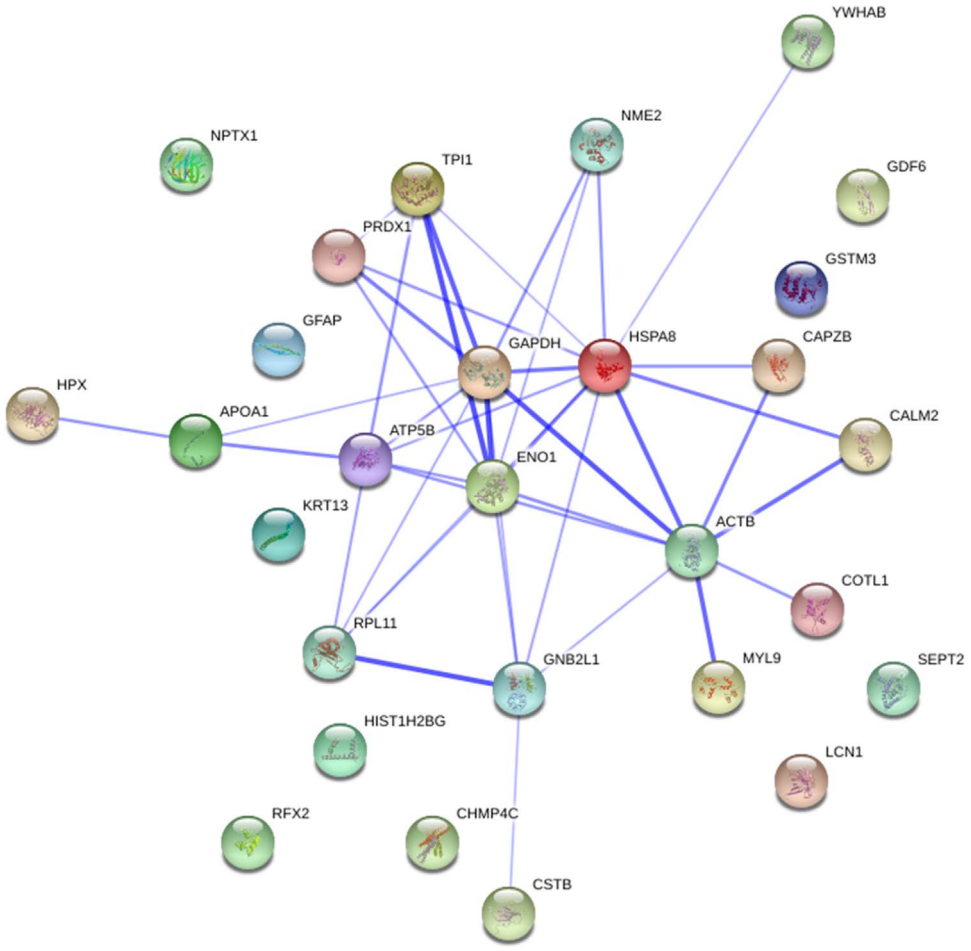

Fig. 3. Confidence view of protein-protein interaction network of identified proteins created by string v9.05. Bolder lines mean higher confidence.

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.

References

[1] G.E. Bledsoe, C.D. Bledsoe, B. Rasco, Caviars and fish roe products, Crit. Rev. Food Sci. Nutr. 43 (2003) 317–356.
[2] A.K. Imsland, P. Reynolds, G. Eliassen, T.A. Hangstad, A.V. Nytrø, A. Foss, et al., Assessment of growth and sea lice infection levels in Atlantic salmon stocked in small-scale cages with lumpfish, Aquaculture 433 (2014) 137–142.
[3] The Health Situation in Norwegian Aquaculture 2014, Fish Health Report Oslo, Norway: National Veterinary Institute Available from, ⟨http://www.vetinst.no/eng/Publications/Fish-Health-Report/Fish-Health-Report-2014⟩. 2015.
[4] M.Á. Esteban, An overview of the immunological defenses in fish skin, ISRN Immunol. 2012 (2012) 1–29.
[5] B. Rajan, J.M.O. Fernandes, C.M.A. Caipang, V. Kiron, J.H.W.M. Rombout, M.F. Brinchmann, Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules, Fish. Shellfish Immunol. 31 (2011) 224–231.
[6] B. Rajan, J. Lokesh, V. Kiron, M.F. Brinchmann, Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*, BMC Vet. Res. 9 (2013) 103.
[7] K. Chong, S. Joshi, L.T. Jin, A.C. Shu-Chien, Proteomics profiling of epidermal mucus secretion of a cichlid (*Symphysodon aequifasciata*) demonstrating parental care behavior, Proteomics 6 (2006) 2251–2258.
[8] J. Jurado, C.A. Fuentes-Almagro, F.A. Guardiola, A. Cuesta, M.Á. Esteban, M.J. Prieto-Álamo, Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*), J. Proteom. 120 (2015) 21–34.
[9] I. Sanahuja, A. Ibarz, Skin mucus proteome of gilthead sea bream: a non-invasive method to screen for welfare indicators, Fish. Shellfish Immunol. 46 (2015) 426–435.
[10] H. Cordero, M.F. Brinchmann, A. Cuesta, J. Meseguer, M.A. Esteban, Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*), Proteomics (2015). http://dx.doi.org/10.1002/pmic.201500120.
[11] R.H. Easy, N.W. Ross, Changes in Atlantic salmon (*Salmo salar*) epidermal mucus protein composition profiles following infection with sea lice (*Lepeophtheirus salmonis*), Comp. Biochem Physiol. D 4 (2009) 159–167.
[12] A.D. Ramos, K. Conceição, P.I. Silva Jr, M. Richardson, C. Lima, M. Lopes-Ferreira, Specialization of the sting venom and skin mucus of *Cathorops spixii* reveals functional diversification of the toxins, Toxicon 59 (2012) 651–665.
[13] H.C. Wang, H.C. Wang, J.H. Leu, G.H. Kou, A.H.J. Wang, C.F. Lo, Protein

expression profiling of the shrimp cellular response to white spot syndrome virus infection, Dev. Comp. Immunol. 31 (2007) 672–686.
[14] A.D. Kulkarni, V. Kiron, J.H.W.M. Rombout, M.F. Brinchmann, J.M.O. Fernandes, N.S. Sudheer, et al., Protein profiling in the gut of *Penaeus monodon* gavaged with oral WSSV-vaccines and live white spot syndrome virus, Proteomics 14 (2014) 1660–1673.
[15] A. Øverbye, M.F. Brinchmann, P.O. Seglen, Proteomic analysis of membrane-associated proteins from rat liver autophagosomes, Autophagy 3 (2007) 300–322.
[16] A. Rogowska-Wrzesinska, M.C. Le Bihan, M. Thaysen-Andersen, P. Roepstorff, 2D gels still have a niche in proteomics, J. Proteom. 88 (2013) 4–13.
[17] S.G. Rhee, H.Z. Chae, K. Kim, Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling, Free Radic. Biol. Med. 38 (2005) 1543–1552.
[18] L. Wang, H. Guo, N. Zhang, Z. Ma, S. Jiang, D. Zhang, Molecular characterization and functional analysis of a peroxiredoxin 1 cDNA from golden pompano (*Trachinotus ovatus*), Dev. Comp. Immunol. 51 (2015) 261–270.
[19] G.H. Loo, D.L. Sutton, K.A. Schuller, Cloning and functional characterisation of a peroxiredoxin 1 (NKEF A) cDNA from Atlantic salmon (*Salmo salar*) and its expression in fish infected with *Neoparamoeba perurans*, Fish. Shellfish Immunol. 32 (2012) 1074–1082.
[20] J. Perez-Sanchez, A. Bermejo-Nogales, J.A. Calduch-Giner, S. Kaushik, A. Sitja-Bobadilla, Molecular characterization and expression analysis of six peroxiredoxin paralogous genes in gilthead sea bream (*Sparus aurata*): insights from fish exposed to dietary, pathogen and confinement stressors, Fish. Shellfish Immunol. 31 (2011) 294–302.
[21] J.R. Riddell, X.Y. Wang, H. Minderman, S.O. Gollnick, Peroxiredoxin 1 stimulates secretion of pro-inflammatory cytokines by binding to Toll-like receptor 4, J. Immunol. 184 (2010) 1022–1030.
[22] G.S. Magalhães, M. Lopes-Ferreira, I.L.M. Junqueira-de-Azevedo, P.J. Spencer, M.S. Araújo, F.C.V. Portaro, et al., Natterins, a new class of proteins with kininogenase activity characterized from *Thalassophryne nattereri* fish venom, Biochimie 87 (2005) 687–699.
[23] S. Tamura, M. Yamakawa, K. Shiomi, Purification, characterization and cDNA cloning of two natterin-like toxins from the skin secretion of oriental catfish *Plotosus lineatus*, Toxicon 58 (2011) 430–438.
[24] M. Baba, B. Yong Ma, M. Nonaka, Y. Matsuishi, M. Hirano, N. Nakamura, et al., Glycosylation-dependent interaction of Jacalin with CD45 induces T lymphocyte activation and Th1/Th2 cytokine secretion, J. Leukoc. Biol. 81 (2007) 1002–1011.
[25] B.Y. Ma, K. Yoshida, M. Baba, M. Nonaka, S. Matsumoto, N. Kawasaki, et al., The lectin Jacalin induces human B-lymphocyte apoptosis through glycosylation-dependent interaction with CD45, Immunology 127 (2009) 477–488.
[26] T.W. Du Clos, Pentraxins: structure, function, and role in inflammation, ISRN Inflamm. 2013 (2013) 22.
[27] S. Tsutsui, M. Yamaguchi, A. Hirasawa, O. Nakamura, T. Watanabe, Common skate (*Raja kenojei*) secretes pentraxin into the cutaneous secretion: the first skin mucus lectin in cartilaginous fish, J. Biochem. 146 (2009) 295–306.
[28] Z. Chen, C.H.C. Cheng, J. Zhang, L. Cao, L. Chen, L. Zhou, et al., Transcriptomic and genomic evolution under constant cold in Antarctic notothenioid fish, Proc. Natl. Acad. Sci. USA 105 (2008) 12944–12949.
[29] N. Yang, C. Peng, D. Cheng, Q. Huang, G. Xu, F. Gao, et al., The over-expression of calmodulin from Antarctic notothenioid fish increases cold tolerance in tobacco, Gene 521 (2013) 32–37.
[30] S. Li, Z. Jia, X. Li, X. Geng, J. Sun, Calmodulin is a stress and immune response gene in Chinese mitten crab *Eriocheir sinensis*, Fish. Shellfish Immunol. 40 (2014) 120–128.
[31] B.L. Lockwood, G.N. Somero, Transcriptomic responses to salinity stress in invasive and native blue mussels (genus *Mytilus*), Mol. Ecol. 20 (2011) 517–529.
[32] P. Sengprasert, P. Amparyup, A. Tassanakajorn, R. Wongpanya, Characterization and identification of calmodulin and calmodulin binding proteins in hemocyte of the black tiger shrimp (*Penaeus monodon*), Dev. Comp. Immunol. 50 (2015) 87–97.
[33] P.F. Ji, C.L. Yao, Z.Y. Wang, Two types of calmodulin play different roles in Pacific white shrimp (*Litopenaeus vannamei*) defenses against Vibrio parahaemolyticus and WSSV infection, Fish. Shellfish Immunol. 31 (2011) 260–268.
[34] M.H. Parseghian, K.A. Luhrs, Beyond the walls of the nucleus: the role of histones in cellular signaling and innate immunity, Biochem Cell Biol. 84 (2006) 589–595.
[35] S. Rakers, L. Niklasson, D. Steinhagen, C. Kruse, J. Schauber, K. Sundell, et al., Antimicrobial peptides (AMPs) from fish epidermis: perspectives for investigative dermatology, J. Invest Dermatol. 133 (2013) 1140–1149.
[36] R. Chen, R. Kang, X.G. Fan, D. Tang, Release and activity of histone in diseases, Cell Death Dis. 5 (2014) e1370.
[37] G. Bergsson, B. Agerberth, H. Jörnvall, G.H. Gudmundsson, Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*), FEBS J. 272 (2005) 4960–4969.
[38] D. Robinette, S. Wada, T. Arroll, M.G. Levy, W.L. Miller, E.J. Noga, Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins, Cell Mol. Life Sci. 54 (1998) 467–475.
[39] W.R. David, J.N. Edward, Histone-like protein: a novel method for measuring stress in fish, Dis. Aquat. Organ. 44 (2001) 97–107.
[40] E. Saitoh, S. Isemura, A. Chiba, S. Oka, S. Odani, A novel cysteine protease inhibitor with lectin activity from the epidermis of the Japanese eel *Anguilla japonica*, Comp. Biochem Physiol. 141 (2005) 103–109.
[41] J. Wynne, M. O'Sullivan, M. Cook, G. Stone, B.F. Nowak, D. Lovell, et al., Transcriptome analyses of amoebic gill disease-affected Atlantic salmon (*Salmo salar*) tissues teveal localized host gene suppression, Mar. Biotechnol. 10 (2008) 388–403.
[42] P.P. Xiao, Y.H. Hu, L. Sun, *Scophthalmus maximus* cystatin B enhances head kidney macrophage-mediated bacterial killing, Dev. Comp. Immunol. 34 (2010) 1237–1241.
[43] J.L. Breslow, D. Ross, J. McPherson, H. Williams, D. Kurnit, A.L. Nussbaum, et al., Isolation and characterization of cDNA clones for human apolipoprotein A-I, Proc. Natl. Acad. Sci. USA 79 (1982) 6861–6865.
[44] M. Kravitz, M. Pitashny, Y. Shoenfeld, Protective molecules-C-reactive protein (CRP), serum amyloid P (SAP), pentraxin3 (PTX3), mannose-binding lectin (MBL), and apolipoprotein A1 (Apo A1), and their autoantibodies: prevalence and clinical significance in autoimmunity, J. Clin. Immunol. 25 (2005) 582–591.
[45] J.W. Pridgeon, P.H. Klesius, Apolipoprotein A1 in channel catfish: transcriptional analysis, antimicrobial activity, and efficacy as plasmid DNA immunostimulant against *Aeromonas hydrophila* infection, Fish. Shellfish Immunol. 35 (2013) 1129–1137.
[46] M.I. Concha, Sa Molina, C. Oyarzún, 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.
[47] D. Aliza, I.S. Ismail, M.K. Kuah, A.C. Shu-Chien, T.S. Tengku Muhammad, Identification of Wap65, a human homologue of hemopexin as a copper-inducible gene in swordtail fish, *Xiphophorus helleri*, Fish. Physiol. Biochem. 34 (2008) 129–138.
[48] A.Y. Gracey, J.V. Troll, G.N. Somero, Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*, Proc. Natl. Acad. Sci. USA 98 (2001) 1993–1998.
[49] Z. Sha, P. Xu, T. Takano, H. Liu, J. Terhune, Z. Liu, The warm temperature acclimation protein Wap65 as an immune response gene: its duplicates are differentially regulated by temperature and bacterial infections, Mol. Immunol. 45 (2008) 1458–1469.
[50] K. Kikuchi, S. Watabe, K. Aida, The Wap65 gene expression of goldfish (*Carassius auratus*) in association with warm water temperature as well as bacterial lipopolysaccharide (LPS), Fish. Physiol. Biochem. 17 (1997) 423–432.
[51] A.G.Pockley, M.Muthana, S.K.Calderwood, The Dual Immunoregulatory Roles of Stress Proteins, Trends Biochem. Sci. vol. 33. pp. 71–79
[52] L. Ribas, J.V. Planas, B. Barton, C. Monetti, G. Bernadini, M. Saroglia, et al., A differentially expressed enolase gene isolated from the gilthead sea bream (*Sparus aurata*) under high-density conditions is up-regulated in brain after in vivo lipopolysaccharide challenge, Aquaculture 241 (2004) 195–206.
[53] D. Kültz, D. Chakravarty, T. Adilakshmi, A novel 14-3-3 gene is osmoregulated in gill epithelium of the euryhaline teleost *Fundulus heteroclitus*, J. Exp. Biol. 204 (2001) 2975–2985.
[54] J. Ulvila, L.M. Vanha-aho, A. Kleino, M. Vähä-Mäkilä, M. Vuoksio, S. Eskelinen, et al., Cofilin regulator 14-3-3ζ is an evolutionarily conserved protein required for phagocytosis and microbial resistance, J. Leukoc. Biol. 89 (2011) 649–659.
[55] D.H. O'Day, R.J. Huber, A. Suarez, Extracellular calmodulin regulates growth and cAMP-mediated chemotaxis in *Dictyostelium discoideum*, Biochem Biophys. Res. Commun. 425 (2012) 750–754.
[56] J. Xu, X. Zhang, R. Pelayo, M. Monestier, C.T. Ammollo, F. Semeraro, et al., Extracellular histones are major mediators of death in sepsis, Nat. Med. 15 (2009) 1318–1321.
[57] F. Descalzi Cancedda, B. Dozin, B. Zerega, S. Cermelli, C. Gentili, R. Cancedda, Ex-FABP, extracellular fatty acid binding protein, is a stress lipocalin expressed during chicken embryo development, Mol. Cell Biochem. 239 (2002) 221–225.
[58] T. Shichita, E. Hasegawa, A. Kimura, R. Morita, R. Sakaguchi, I. Takada, et al., Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain, Nat. Med. 18 (2012) 911–917.
[59] N. Asdaghi, R. Kilani, A. Hosseini Tabatabaei, S.O. Odemuyiwa, T.L. Hackett, D. Knight, et al., Extracellular 14-3-3 from human lung epithelial cells enhances MMP-1 expression, Mol. Cell Biochem. 360 (2012) 261–270.
[60] A. De Maio, Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: a form of communication during injury, infection, and cell damage, Cell Stress Chaperon. 16 (2011) 235–249.
[61] M.F. Brinchmann, Immune relevant molecules identified in the skin mucus of fish using-omics technologies, Mol. Biosyst. 12 (2016) 2056–2063.

Paper II

Title: Proteomic and structural differences in lumpsucker skin among the dorsal, caudal and ventral regions

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Fish skin is a vital organ that serves a multitude of functions including mechanical protection, homeostasis, osmoregulation and protection against diseases. It contains proteins involved in immune response serving as first line of defence. The expression of skin proteins changes under different physiological conditions. However, little is known about differences in protein expression among various body sites in fish. Objectives of this study is to find differentially expressed proteins among dorsal, caudal and ventral regions of lumpsucker skin employing 2D gel based proteomics and to find structural differences between these regions by using periodic acid-Schiff/alcian blue stained skin sections. Collagen alfa-1, collagen alfa-2, heat shock cognate 71 kDa, histone H4, parvalbumin, natterin-2, 40S ribosomal protein S12, topoisomerase A and topoisomerase B were differentially expressed among the three regions. Skin photomicrographs showed differences in epidermal thickness and goblet cell counts. The ventral region showed relatively high protein expression, goblet cell count and epidermal thickness compared to dorsal and caudal regions. This could be to provide energy and strength to adhere to objects in its habitat through the ventral disc. Over all this study provides important benchmark for comparative analysis of fish skin proteins and structure among different regions in fish body.

Introduction

Skin is the largest and outermost organ in the fish body. It is the first protective barrier between its internal organs and the external aquatic environment [1]. Teleost skin is mainly composed of two layers, the outer epidermis and inner dermis. In addition to epidermis and dermis, fish skin possesses an epidermal mucus layer that acts as primary line of defence against pathogens [2]. Apart from that, the skin also serves as a barrier against physical abrasion, environmental toxins and physiological stress responses [3]. Knowledge on fish skin, its associated proteins, their function and response to various stimuli helps to understand the skin associated defence mechanism. It is not only important for fish health and welfare, but it is also important in mucosal immune research on higher vertebrates as it shares many features with mammalian gut [2]. Hence, fish skin and its mucosal factors have been one of the major topics of research since decades.

The species of interest in this study is *Cyclopterus lumpus*, also known as lumpfish/lumpsucker. It is a scorpaeniform fish distributed throughout the Norwegian Sea. This fish is widely used as a biological tool for sea lice removal in Atlantic salmon farms [4]. Although lumpsucker is very popular as a powerful weapon against sea lice, this species is poorly studied in terms of its biology and immune defence system. Thus, a better understanding on the biology of this species is needed to cope with arising risk factors in lumpsucker farming due to disease causing agents. Skin associated innate immune response play an important role in defence against diseases [5]. To study the complex immune network of fish skin, and its interaction with disease causing factors, it is essential to identify and explore its associated elements at molecular level. To achieve this, omics technologies provide a suitable platform to explore the skin associated defence factors [6]. Two dimensional gel based proteomics have been a suitable tool to identify and study proteins of non-model species that have very limited gene and protein information available in various databases [7]. We have earlier identified proteins in lumpsucker skin mucus [8] among these some of the proteins have been found in other teleost species and been shown to have a role in immune response [1]. Skin/skin mucus proteomic analysis has also been performed in other teleost species such as healthy [9] and *Vibrio anguillarum* infected Atlantic cod (*Gadus morhua*) [10], haelthy European sea bass (*Dicentrarchus labrax*) [11], healthy gilthead sea bream (*Sparus aurata*) [12], probiotic fed [13],

and chronically stressed gilthead sea bream [14], epidermal mucus proteomic analysis of cichlid (*Symphysodon aquifasciatus*) to asses parental care [15], sea lice infected Atlantic salmon (*Salmo salar*) [16], *Aeromonas hydrophila* infected zebrafish (*Danio rerio*) [17], and marine catfish (*Cathorops spixii*) [18].

The present study aims to identify skin proteins in lumpsucker and their expression levels in different regions of skin by employing a 2D gel based proteomics method. To our knowledge this is the first study approaching a differential protein expression among various body sites in fish.

Results

Mass spectrometry analysis of lumpsucker skin samples

In this study we have identified proteins in lumpsucker skin by using 2D gel electrophoresis coupled with liquid chromatography and tandem mass spectrometry. This method along with homology search is a very useful tool for proteome analyses of species like lumpsucker with very little molecular data available in public databases. A total of 18 gels, 3 gels per each of 6 fish (one gel from each region, i. the dorsal region (D); above the lateral line close to operculum, ii. the caudal region (C); below the lateral line close to caudal peduncle, iii. the ventral region (V); near the adhesive disc) were run and electronic images were used for PDQuest analysis (BioRad) (Fig 1). Seventeen differentially expressed spots among the three different skin regions were excised, subjected to LC-MS/MS and identified using MASCOT. Of 17, 10 spots gave specific protein hits and were identified, the rest remain unidentified (Fig 2 and 3). In addition to the differentially expressed spots we also excised 83 spots that were expressed in all three regions in high density (Fig 2). Details of identified proteins are listed in Table 1. Proteins that had significant differential expression among the three different skin regions are shown in Fig 3. All gel images are provided as Supplementary Fig 1.

Gene ontology and protein interaction

Gene ontology terms of biological process of the identified proteins were retrieved manually from UniProt. As most of the proteins are not well annotated in teleost species the gene ontology terms were retrieved from its human counterparts (Table 1).

A possible protein-protein interaction map was created employing zebrafish orthologues with high edge confidence level (<0.700) using string v10.0 (Fig.4). The protein interaction network created 45 nodes and 39 edges with an average node degree of 1.73. The network highlights the interaction of ribosomal proteins (rpsa, rplp0, rps12, rpl18, rps25), histone proteins (hist1h41, cr762436.3, LOC560309), cytoskeletal proteins (actc1, acta1b, tpma, cfl2l, cfl1), enzymes (ak1, mdh2, gapdh, tpi1a, tpi1b, atp5h), nucleotide binding proteins (gnb1a, zgc:110283), proteasome subunits (psmb1, psmb2), parvalbumin, apolipoprotein A1 and transferrin. Full protein names of the abbreviations used in the protein interaction network are provided as Supplementary Table S1. All abbreviations of the protein in the interaction map are assigned by string.

mRNA expression levels of immune and/or stress genes in lumpsucker skin

A few genes known to have immune and/or stress responses in other fish species were selected for real time quantitative PCR analysis to gain additional knowledge on their possible differential expression in different skin regions. Three reference genes (*gapdh, β-actin, ef1-alfa*) and four target genes (*apoa1, hspa8, calm, hist1h2b*) were selected for this study. Due to unavailability of lumpsucker gene sequences in various databases we designed the primers using sequences from other teleost species focusing on conserved regions. The primers for reference genes did not have degeneracy and was used for the real time assay (Table 2). But the target genes showed degeneracy (Table 2) and had to be sequenced. PCR products obtained by using degenerate primers were verified by subsequent sequencing of amplified fragments. These sequences were used for real time PCR primer designing for target genes (Table 2).

PCR products obtained by using real time primers for both reference and target genes were sequenced for verification of identity. All genes were amplified for melt curve analysis. A single peak was obtained in most of the genes whereas in a few a small peak of primer dimer was detected that was also observed in the negative control. Negative controls did not show any amplification.

All three reference genes showed a Cq value ranging from 18-24. BestKeeper[19] software was used to analyse the expression stability of candidate reference genes. As per the analysis the coefficient of correlation (*r*) of *gapdh, β-actin, ef1-alfa* genes are 0.930, 0.882, 0.865 respectively.

The detailed analysis obtained from BestKeeper are available as Supplementay Table S2. As all three genes did not show much variation in their expression pattern, the geometric mean of Cq obtained from *gapdh, β-actin* and *ef1-alfa* was used to analyse the relative expression level of target genes.

The expression patterns of selected target genes (*apoa1, hspa8, calm, hist1h2b*) for real time quantitative PCR are shown in Fig 5. Significant differential expression of *apoa1* mRNA was detected in the ventral region compared to the dorsal and caudal regions. *calm* mRNA did not show any significant difference among the different regions of skin. *hspa8* mRNA expression followed the protein expression pattern showing high expression in caudal region followed by dorsal and ventral region. *hspa8* mRNA expression in the ventral region was found significantly different from the dorsal region. However, it did not show any significant difference between dorsal and caudal region. *hist1h2b* mRNA expression in caudal region was found to be significantly different from the ventral region where as there was no significant difference observed between dorsal and ventral region.

Histological findings

The histological analysis of lumpsucker skin showed that epidermal thickness (Fig 6e) and goblet cell count (Fig 6d) were significantly higher in the ventral region compared to the dorsal and caudal regions. Goblet cells were predominantly found towards the outer layer of epidermis. In the dorsal and caudal region we observed many cells similar to saccular cells (Fig 6a, 6b), but these cells were absent in ventral region (Fig 6c). Melanin deposition was observed in upper layer of dermis in dorsal and caudal region (Fig 6a, 6b), but not in the vertical region.

Discussion

Skin is a vital organ in fish that serves an array of functions to maintain homeostasis. It contains various immune related proteins such as antimicrobial proteins, lectins, immunoglobulins, complement factors, proteases, and acute phase proteins [6]. In this study we have identified several differentially expressed proteins in skin of lumpsucker among three different regions (D, C, V). There are very few studies that have focused on differences among different regions in fish skin at the molecular level. A study on

Atlantic cod (*G. morhua*) reported the dorso-ventral differential expression of several immune and stress related genes in skin tissue of healthy fish [20]. Furthermore, another study showed difference in expression levels of some immune genes in skin cells of Atlantic cod isolated from dorsal and ventral regions after probiotics-pathogen interactions [21]. Expression of agouti gene have been evaluated to study the dorso-ventral pigmentation pattern in gold fish (*Carrasius auratus*) using northern blot [22], and in flatfishes (*Scopthalmus maximus* and *Solea senegalensis*) using quantitative real time PCR [23]. However, no study has been carried out to assess the difference of protein expression among various regions in fish skin.

In the present study among the differentially expressed spots, D14, D35 and D95 were identified as collagen alpha-1 and alpha-2 type proteins. Collagen has been isolated from skin of several teleost species for industrial purposes as an alternative to pig and bovine collagen [24-27]. Collagen is an essential extra cellular matrix protein in fish [28] that provides mechanical support to maintain skin integrity and is also involved in immune responses. An *in vitro* study on gilt head sea bream found that collagen could prime respiratory burst and regulate the phagocytic activity [29]. In this study, the alpha-1 and alpha-2 type collagen proteins, which are essential for formation of type I collagen showed relatively higher expression in the ventral region than in the dorsal and caudal regions (Fig 3). This expression could be due to the presence of the adhesive disc (modified pelvic fin) in the ventral region of lumpsucker. This fish spends most of its time as a sessile organism by adhering to suitable objects rather than swimming actively. The adhesive disc in this species has very strong adhesion capacity to protect the fish against the water current. Therefore, high abundance of protein like collagen could provide mechanical strength to maintain the balance and skin integrity against strong water currents.

Spot D15 was identified as natterin, a protein with lectin like domain and a toxic domain with kinogenase activity [30]. In skin, the lectin like domain could recognise pathogens and the toxin domain could cause lysis of pathogenic microbes. This protein was purified with a mannose affinity column from Atlantic cod skin mucus that suggests its mannose specific domain could act as pathogen recognition receptor in the skin [31].

Spot D24 was recognized as histone H4. Histones along with their primary nuclear functions (Fig 4) also serve as danger associated molecular patterns when released to

extracellular space. This group of protein are also involved in inflammation, cell death and immune responses [32]. Histone H4 from fresh water prawn (*Macrobrachium rosenbergii*) showed antimicrobial activity against both Gram negative and Gram positive bacteria. Furthermore, high H4 gene expression levels were reported in gills of fresh water prawn infected with pathogens such as white spot syndrome baculovirus, *M. rosenbergii* noda virus, *A. hydrophila* and *Vibrio harveyi* [33]. Histone proteins have been identified in skin/skin mucus of several teleosts such as histone H4 in mrigal (*Cirrhinus mrigala*) [34] and European seabass (*D. labrax*) [11], H2B like protein in channel catfish (*Ictalurus punctatus*) [35], histone like protein in sunshine bass (*Morone saxatilis*) [36], histone like protein and H2A in rainbow trout (*Onchorynchus mykiss*) [37,38], histone derived antimicrobial peptides in Atlantic halibut (*Hippoglossus hippoglossus*) [39] and coho salmon (*Onchorynchus kisutch*) [40], and histone H2B in lumpsucker [8]. In addition to histone H4, H2A (spot D21) and H2B (spot D23) were also identified in lumpsucker skin in this study, but these two proteins did not show any differential expression among different skin regions.

Spot D25 and D68 were identified as parvalbumin. This protein is a widely studied fish allergen [41,42] however very little is known about its role in host defence. Pravalbumin is a calcium binding protein and hence it could inhibit bacterial growth by chelating the essential cations needed for the growth and proliferation of bacteria. This hypothesis was confirmed by a study on parvalbumin extracted from cutaneous mucus of Thamnophiine snake (*Lithobates catesbeianus*) showing antibacterial activity against *Escherchia coli* [43]. This protein is involved in intercellular calcium binding that might function in calcium ion transport during muscle relaxation [44] in association with other cytoskeletal proteins as shown in (Fig 4). Parvalbumin is also widely distributed throughout the nervous system. A study on rat visual cortex during postnatal development revealed that expression level of parvalbumin is dependent on the neuronal activity where they found a positive correlation between number of neurons and parvalbumin expression [45]. Thus, high level of parvalbumin expression in ventral region than dorsal and caudal region in skin of lumpsucker (Fig 3) could be due to the presence of adhesive disc, as skin surrounding the disc needs to be neurologically alert. Relatively high level of parvalbumin, histone H4 in ventral region of lumpsucker skin (Fig 3) could protect it from pathogenic microbes when

217 the adhesive disc is in close contact with surfaces that may contain disease causing
218 agents.
219 Spot D42 was heat shock cognate 71kDa protein (hsc71). This protein is a member of the
220 highly conserved heat shock protein 70 family [46]. This is a multifunctional protein that
221 acts as molecular chaperone, stress indicator and signalling molecule [47,48]. Presence of
222 this protein/gene has been reported in skin mucus of sea lice infected Atlantic salmon
223 analysed by microarray [49], and skin mucus of naïve lumpsucker analysed by 2D gel based
224 proteomics [8]. Expression of this protein/gene is up-regulated during stress induced by
225 environmental parameters [50]. Heat shock cognate 71 gene in catfish (*Clarius batrachus*)
226 showed relatively higher level of expression in different tissues (brain, muscle, spleen,
227 heart, liver, head kidney) under hypoxic condition than the control fish that were
228 maintained under normal oxygen level [50]. In human hsc71 has found to be interacting
229 with MHC molecules and be involved in regulation of antigen trafficking [47]. Heat shock
230 cognate proteins serves as a link between chaperones and the proteasome [51] for
231 proteasome activation for degradation of misfolded proteins (Fig 4).
232 Spot D45 and D89 were identified as triosephosphate isomerase B and A respectively.
233 These enzymes are involved in carbohydrate metabolism and isomerisation of
234 dihydroxyacetone phosphate into glyceraldehyde-3-phosphate. Triosephosphate
235 isomerases have been reported in skin mucus of lumpsucker [8], Atlantic cod [9], gilthead sea
236 bream [12,13], and European sea bass [11]. This protein was found to be significantly up-
237 regulated in early developmental stages of mussel (*Mytilus galloprovincialis*) due to
238 oxidative stress induced by cadmium, where the authors concluded that up-regulation of
239 triosephosphate isomerase could be for compensation of the energy demand induced by
240 stress [52]. Relatively high expression of these proteins in ventral region of lumpsucker skin
241 (Fig 3) in this study could be due to comparatively high energy demand for successful
242 adhesion to various substrates.
243 In addition to differentially expressed proteins, the present study also identified abundant
244 proteins in lumpsucker skin. We have previously reported various immune and/or stress
245 related proteins in skin mucus of lumpsucker [8]. Several proteins that were reported in
246 skin mucus are also identified in skin in the present study. Spot D18 was identified as
247 calmodulin. This protein is involved in inflammatory responses, intracellular and

extracellular signalling [53] and stress responses [54,55]. Spots D30, D31, and D32 were identified as apolipoprotein A1. This protein is primarily involved in transportation of high-density lipoprotein particles. Antimicrobial activity of apolipoprotein A1 isolated from plasma was observed in common carp (*Cyprinus carpio*) against both Gram negative and Gram positive bacteria [56]. Increased expression of this protein was observed in skin mucus of Atlantic salmon infected by sea lice [16] and in gill mucus of Atlantic salmon affected by amoebic gill disease [57]. Spots D43 and D44 were identified as transferrin and serotransferrin. Transferrin is well known for its role in transfer and delivery of iron to the cells. It binds to iron and makes it unavailable for bacteria and creates a bacteriostatic environment. This protein is also found to be an activator of macrophagic activity by inducing nitric oxide response in macrophages in goldfish exposed to several fish pathogens [58]. Furthermore, cleaved transferrin has found to be involved in acute inflammatory responses in goldfish injected with heat killed *Aeromonas veronii* [59]. Spot D96 was identified as natural killer enhancing factor. This protein is also known as peroxiredoxin, an antioxidant protein involved in immune responses in fish such as chaperoning, inflammatory responses upon infection, balance of reactive oxygen production to reduce oxidative stress [60].

After protein identification, quantitative real time PCR has been widely used as a complementary technique to analyse whether local syntheses of the proteins are possible or not [61]. Due to unavailability of gene sequences of lumpsucker for primer design, we designed degenerate primers by focusing on conserved regions in order to reduce degeneracy. This technique is cost effective and promising for designing primers for new species with no available information. However, this could fail to amplify if the degeneracy level is too high. Therefore, we could get successful amplification of only few genes. Sanger sequencing of the amplified products from degenerate PCR not only confirmed the identity of the genes but also provided the sequence for designing real time PCR primers.

Some of the mRNA expression pattern did not follow the protein expression in this study. Similar results has been obtained in Atlantic cod challenged with *V. anguillarum* [62] and in yeast (*Saccharomyces cerevisiae*) [63] where the proteomic data did not correlate with the gene expression data. There are number of factors that affects the mRNA-protein

correlation such as the secondary structure of mRNA which changes continuously or under certain conditions affecting the translation efficiency, regulatory proteins could repress translation, codon bias and ribosomal density affects the translation of proteins, protein half lives after post translational modifications also serves as a major factor influencing the mRNA-protein correlation [64].

Periodic acid-Schiff and alcian blue stained skin sections of lumpsucker observed by light microscopy showed thicker epidermis in the ventral region than the dorsal and caudal regions. Similar results were observed in a study conducted on gilthead sea bream (*Sparus aurata*) with thicker epidermis in ventral region than dorsal [65]. In benthic species the ventral epidermis is often thicker than other regions [66]. Thicker ventral epidermis in lumpsucker could be due to the sedentary nature of the fish while adhering to the substrates in its habitat. Goblet cells (mucus producing cells) are important features of fish epidermis and are found in most of the teleost species. These cells vary in numbers depending on the location in the body, sex, life stages and physiological conditions such as infection [1]. We observed relatively higher numbers of goblet cells in the ventral region than the dorsal and caudal regions. This could be to produce more mucus to provide a protective layer against pathogens. Lumpsucker skin sections showed a dermal pigment deposition in dorsal and caudal regions but not in the ventral region. Pigment cells have been seen in many teleost species (both scale and scaleless) in the dermal layer [67]. We identified several cells similar to saccular cells in Atlantic halibut [68]. These cells appeared as single vacuole in the epidermis in dorsal and caudal regions only. These cells did not respond to alcian blue (pH-2.5) stain as suggested by Mittal et al. [69] and Ottesen et al. [68].

Conclusion

In the present study we used 2D gel based proteomics and LC-MS/MS to identify differentially expressed proteins among the dorsal, caudal and ventral regions of lumpsucker skin. Our results indicated that some of the proteins showed differential expression between these three body sites. Using light microscopy we observed structural differences among the three regions of skin in terms of epidermal thickness, goblet cell counts and saccular cells. The epidermal thickness and goblet cell count was relatively more in ventral region than the other two regions. But we did not observe saccular cells

in ventral region. This is the first study to report differences of protein expression among different parts of skin in fish. It could provide a platform for quantitative comparison of skin proteome under various physiological conditions focusing on specific body sites. All together this study provides a sound knowledge about lumpsucker skin structure and its associated molecular factors.

Materials and methods

Fish rearing and tissue sampling

Lumpsucker larvae (2 dph) were obtained from Arctic Cleanerfish, Stamsund, Norway and reared at Mørkvedbukta Research Station, Nord University, Bodø, Norway. Larvae were raised in 80 litre capacity black circular tanks with flow through seawater at 10-12°C. All fish were fed with commercial diet from Skretting, Norway. Lumpsuckers (n = 6) of approximate weight 60-70 g were anaesthetized in MS-222 (70 mg/l) and humanely killed by giving a blow to the head. Skin samples from three different regions (D, C, V) were sampled. For proteomic analysis the tissues were snap frozen in liquid nitrogen and for real time PCR the tissues were put in RNA later. All samples for proteomics and real time PCR were moved to -80°C until further analysis. Skin tissues for histology were fixed in 10% phosphate buffered formaldehyde solution. All animal rearing and handling procedures were performed according to guidelines set by National Research Authority, Norway.

Two-dimensional gel electrophoresis

Frozen skin samples were homogenized with liquid nitrogen by using pestle and mortar. Homogenized tissue was mixed with 1x PBS containing protease inhibitor (GE Healthcare Life Sciences) and sonicated two times (5 s each with an interval of 1 min) on ice using an ultrasonic processor (SONICS Vibracell VCX750, USA). The sonicated sample was centrifuged at 15,000 g for 30 min at 4°C to pellet the debris in the tissue sample. The resulted supernatant was processed as described in [8]. Protein was quantified using Qubit Fluorometer, Invitrogen. IPG strips (pH 3-10, 17cm, BioRad, USA) were rehydrated with 100 µg of protein and electro focused. Electro focused strips were run on 12.5% polyacrylamide gels for approximately 16 h and stained in SYPRO® Ruby (ThermoFisher Scientific, USA) fluorescent protein stain. Gel images were documented

using ChemiDoc® XRS system (BioRad, USA) and used for PD Quest (BioRad, USA) analysis.

LC-MS/MS and protein identification

Abundant and/or spots with different expression levels among three regions (D, C, V) were excised and subjected for LC-MS/MS analysis. LC-MS/MS analysis was performed at University of Tromsø, Norway by using nanoACQUITY ultra performance liquid chromatography system and Q-TOF mass spectrophotometer (Micromass/Waters, MA, USA). The peak list files generated from LC-MS/MS analysis were analysed by MASCOT MS/MS Ion search (version 2.5). A homology driven search was performed using various protein databases including SwissProt (553941 sequences; 198311666 residues, March 2017) and NCBInr (116205035 sequences; 42603624384 residues, March 2017) for protein identification. Parameters set for identification were carbamidomethyl (C) fixed modification, oxidation (M) variable modification, monoisotopic, peptide charge 2+ and 3+, enzyme trypsin with maximum 1 missed cleavage, peptide tolerance 100 ppm and MS/MS tolerance 0.1 Da. The identification was restricted to taxonomic group Actinopterygii keeping the false discovery rate below 1%. Proteins showing significant hits ($p<0.05$) with a score above threshold level and at least one unique peptide sequence were identified.

Gene ontology and protein-protein interaction

Gene ontology terms of identified proteins were retrieved from their human orthologues from UniProt KB protein database according to their biological process. The protein-protein interaction map was constructed using string v.10 with a high edge confidence limit. Zebrafish orthologues of identified proteins were used as input for protein protein interaction analysis due to unavailability of species-specific data for lumpsucker.

RNA extraction and cDNA preparation

Total RNA from lumpsucker skin was extracted by using E.Z.N.A.® Total RNA Kit (Omega Bio-tek, Norcross, GA) following the manufacturer's protocol. RNA integrity was determined by observing two distinct bands representing 18S and 28S on 1% agarose gel. RNA was quantified using Qubit RNA BR assay kit and the Qubit Fluorometer (ThermoFisher Scientific, USA). The extracted RNA was reverse transcribed to synthesize cDNA from 1µg of total RNA using QuantiTect reverse transcription kit

(Qiagen, Germany) as described by the manufacturer. The cDNA samples were 50x diluted for qPCR analysis.

Primer design

Till date lumpsucker genome has not been sequenced and also the nucleotide sequences of the selected proteins are not available in the databases. Therefore, degenerate primers were designed using geneious9 software (Biomatters, New Zealand) and restriction sites (GCTGGCGCCTCTCTAGACACAGGATCC for forward and GTCGACAAGGGTACCATAGAAGGGAGAAGC for reverse) were added to each primer. PCR amplification for degenerate primers were performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 34 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min, and final extension at 72°C for 2 min. The PCR products were ran on 1% agarose gel. Expected bands from the gel were excised and DNA was purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany). Purity and concentration of gel purified DNA was analysed by Nanodrop 1000, (ThermoFisher Scientific, USA). Further, the purified DNA was sequenced using ABI 3100 DNA sequencer, (Applied Biosystems, USA) using Big dye termination chemistry (ThermoFisher Scientific, USA). Sequences obtained from the DNA sequencer were used for real time primer designing. The oligonucleotide sequences and specifications are mentioned in Table 2. All primers used for real time analysis were sequenced and blasted against NCBI to confirm their identification.

Quantitative real time PCR

Three reference genes *gapdh*, *ef1-alfa* and *β-actin* were selected for the study. The Excel based tool BestKeeper [19] was used to analyse stability of the genes. Real time quantitative PCR was performed on Applied Biosystems Step OnePlus using SYBR green chemistry (Applied Biosystems, USA).

Standards were prepared to generate calibration curve for estimation of PCR efficiency of primers. Total RNA samples were pooled and reverse transcribed to make cDNA for preparation of standards. Five series of three fold dilutions (1:3, 1:9, 1:27, 1:81, 1:243, 1:729) were prepared from undiluted cDNA. The cDNA from each dilution was further diluted to 1:3 dilutions with molecular grade water. PCR efficiency (E) for each primer was calculated according to formula E = 10^(-1/slope) [70].

All gene amplifications were performed in a total volume of 10 µl containing 5 µl of SYBR® green PCR master mix, 4 µl of template DNA and 1 µl of primer mix (5 µM of each forward and reverse primers). The amplification condition involves a holding stage for 20 s at 95°C followed by 35 cycles of denaturation at 95°C for 3 s, annealing at 60°C, for 30 s and during each cycle at the annealing stage data acquisition step was included for 15 s at 60°C. All plates were run with negative controls (no template control and no reverse transcriptase control) and positive control (pooled cDNA from all samples). All reactions were carried out in triplicates. The qPCR data was analysed by employing one-way ANOVA (analysis of variance) followed by Tukey HSD (honest significant difference) post hoc analysis ($p<0.05$, $n = 6$).

Histology

Lumpsucker skin tissues of approximately 0.5 cm^2 from different regions (D, C, V) were sampled (n=5), immediately fixed in 10% phosphate buffered formaldehyde solution and left at room temperature for 24 h. The samples were dehydrated using a standard histological technique with a series of graded ethanol treatments, embedded in paraffin and sectioned into 4 µm sections. Skin sections were stained with a combination of 1% periodic acid-Schiff and alcian blue (pH 2.5) stain. Photomicrographs of skin sections were prepared using light microscopy and Cell B imaging software (Olympus, Germany). Goblet cells were counted for each region from five fishes. For goblet cell counting three equal sized area from each section were randomly selected and cells were counted using manual settings in Fiji software v2.0.0 [71]. Thickness of skin epidermis of the three regions was measured. Both thickness measurements and goblet cell counts were statistically analysed using ANOVA and Tukey HSD post hoc analysis ($p < 0.05$).

References

1 Esteban, M. A. An Overview of the immunological defenses in fish skin. *ISRN Immunol.* **2012**, 1-29 (2012).

2 Xu, Z. *et al.* Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *P. Natl. Acad. Sci. USA* **110**, 13097-13102 (2013).

3 Bols, N. C., Brubacher, J. L., Ganassin, R. C. & Lee, L. E. J. Ecotoxicology and innate immunity in fish. *Dev. Comp. Immunol.* **25**, 853-873 (2001).

4 Imsland, A. K. *et al.* Assessment of growth and sea lice infection levels in Atlantic salmon stocked in small-scale cages with lumpfish. *Aquaculture* **433**, 137-142 (2014).

5 Magnadóttir, B. Innate immunity of fish (overview). *Fish Shellfish Immunol.* **20**, 137-151 (2006).

6 Brinchmann, M. F. Immune relevant molecules identified in the skin mucus of fish using -omics technologies. *Mol. Biosyst.* **12**, 2056-2063 (2016).

7 Rogowska-Wrzesinska, A., Le Bihan, M. C., Thaysen-Andersen, M. & Roepstorff, P. 2D gels still have a niche in proteomics. *J. Proteomics* **88**, 4-13 (2013).

8 Patel, D. M. & Brinchmann, M. F. Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). *Biochem. Biophys. Rep.* **9**, 217-225 (2017).

9 Rajan, B. *et al.* Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. *Fish Shellfish Immunol* **31**, 224-231 (2011).

10 Rajan, B., Lokesh, J., Kiron, V. & Brinchmann, M. F. Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*. *BMC Vet Res* **9**, 103 (2013).

11 Cordero, H., Brinchmann, M. F., Cuesta, A., Meseguer, J. & Esteban, M. A. Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). *Proteomics*, doi:10.1002/pmic.201500120 (2015).

12 Jurado, J. *et al.* Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*). *J. Proteomics* **120**, 21-34 (2015).

13 Cordero, H., Morcillo, P., Cuesta, A., Brinchmann, M. F. & Esteban, M. A. Differential proteome profile of skin mucus of gilthead seabream (*Sparus aurata*) after probiotic intake and/or overcrowding stress. *J. Proteomics* **132**, 41-50 (2016).

14 Pérez-Sánchez, J. *et al.* Skin mucus of gilthead sea bream (*Sparus aurata* L.). Protein mapping and regulation in chronically stressed fish. *Front. Physiol.* **8**, 34 (2017).

15 Chong, K., Joshi, S., Jin, L. T. & Shu-Chien, A. C. Proteomics profiling of epidermal mucus secretion of a cichlid (*Symphysodon aequifasciata*) demonstrating parental care behavior. *Proteomics* **6**, 2251-2258 (2006).

16 Easy, R. H. & Ross, N. W. Changes in Atlantic salmon (*Salmo salar*) epidermal mucus protein composition profiles following infection with sea lice (*Lepeophtheirus salmonis*). *Comp. Biochem. Phys. D* **4**, 159-167 (2009).

17 Lü, A. *et al.* Proteomic analysis of differential protein expression in the skin of zebrafish [*Danio rerio* (Hamilton, 1822)] infected with *Aeromonas hydrophila*. *J. Appl. Ichthyol.* **30**, 28-34 (2014).

18 Ramos, A. D. *et al.* Specialization of the sting venom and skin mucus of *Cathorops spixii* reveals functional diversification of the toxins. *Toxicon* **59**, 651-665 (2012).

19 Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **26**, 509-515 (2004).

20 Caipang, C. M. A., Lazado, C. C., Brinchmann, M. F., Rombout, J. H. W. M. & Kiron, V. Differential expression of immune and stress genes in the skin of Atlantic cod (*Gadus morhua*). *Comp. Biochem. Phys. D* **6**, 158-162 (2011).

21 Lazado, C. C. & Caipang, C. M. A. Probiotics pathogen interactions elicit differential regulation of cutaneous immune responses in epidermal cells of Atlantic cod *Gadus morhua*. *Fish Shellfish Immunol.* **36**, 113-119 (2014).

22 Cerdá-Reverter, J. M., Haitina, T., Schiöth, H. B. & Peter, R. E. Gene structure of the goldfish agouti-signaling protein: A putative role in the dorsal-ventral pigment pattern of fish. *Endocrinology* **146**, 1597-1610 (2005).

23 Guillot, R., Ceinos, R. M., Cal, R., Rotllant, J. & Cerdá-Reverter, J. M. Transient ectopic overexpression of agouti-signalling protein 1 (Asip1) induces pigment anomalies in flatfish. *PloS One* **7**, e48526, doi:10.1371/journal.pone.0048526 (2012).

24 Nagai, T., Araki, Y. & Suzuki, N. Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chem.* **78**, 173-177 (2002).

25 Tang, L. *et al.* Physicochemical properties and film-forming ability of fish skin collagen extracted from different freshwater species. *Process Biochem.* **50**, 148-155 (2015).

26 Virginia, A., Rachmawati, H., Riani, C. & Retnoningrum, S. D. Study of HMG-CoA reductase inhibition activity of the hydrolyzed product of snakehead fish (*Channa striata*) skin collagen with 50 kDa collagenase from *Bacillus licheniformis* F11.4. *Sci. Pharm.* **84**, doi:10.3797/scipharm.ISP.2015.01 (2016).

27 Kimura, S., Ohno, Y., Miyauchi, Y. & Uchida, N. Fish skin type I collagen: wide distribution of an α3 subunit in teleosts. *Comp. Biochem. Phys. B* **88**, 27-34 (1987).

28 Gistelinck, C. *et al.* Zebrafish Collagen Type I: Molecular and biochemical characterization of the major structural protein in bone and skin. *Sci. Rep.* **6**, 21540, doi:10.1038/srep21540 (2016).

29 Castillo-Briceño, P. *et al.* Collagen regulates the activation of professional phagocytes of the teleost fish gilthead seabream. *Mol. Immunol.* **46**, 1409-1415 (2009).

30 Magalhães, G. S. *et al.* Natterins, a new class of proteins with kininogenase activity characterized from *Thalassophryne nattereri* fish venom. *Biochimie* **87**, 687-699 (2005).

31 Rajan, B., Patel, D. M., Kitani, Y., Viswanath, K. & Brinchmann, M. F. Novel mannose binding natterin-like protein in the skin mucus of Atlantic cod (*Gadus morhua*). *Fish Shellfish Immunol.* **68**, 452-457 (2017).

32 Chen, R., Kang, R., Fan, X. G. & Tang, D. Release and activity of histone in diseases. *Cell Death Dis.* **5**, e1370, doi:10.1038/cddis.2014.337 (2014).

33 Chaurasia, M. K. *et al.* A prawn core histone 4: Derivation of N- and C-terminal peptides and their antimicrobial properties, molecular characterization and mRNA transcription. *Microbiol. Res.* **170**, 78-86 (2015).

34 Nigam, A. K., Kumari, U., Mittal, S. & Mittal, A. K. Evaluation of antibacterial activity and innate immune components in skin mucus of Indian major carp, *Cirrhinus mrigala*. *Aquac. Res.* **48**, 407-418 (2017).

35 Robinette, D. *et al.* Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. *Cell Mol. Life Sci.* **54**, 467-475 (1998).

36 Noga, E. J., Fan, Z. & Silphaduang, U. Histone-like proteins from fish are lethal to the parasitic dinoflagellate *Amyloodinium ocellatum*. *Parasitology* **123**, 57-65 (2001).

37 Edward, J. N., Zhiqin, F. & Umaporn, S. Host site of activity and cytological effects of histone like proteins on the parasitic dinoflagellate *Amyloodinium ocellatum*. *Dis. Aquat. Organ.* **52**, 207-215 (2002).

38 Fernandes, J. M. O., Kemp, G. D., Molle, M. G. & Smith, V. J. Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Biochem. J.* **368**, 611-620 (2002).

39 Birkemo, G. A., Lüders, T., Andersen, Ø., Nes, I. F. & Nissen-Meyer, J. Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). *BBA- Proteins and Proteomics* **1646**, 207-215 (2003).

40 Patrzykat, A., Zhang, L., Mendoza, V., Iwama, G. K. & Hancock, R. E. W. Synergy of histone derived peptides of coho salmon with lysozyme and flounder pleurocidin. *Antimicrob. Agents. Ch.* **45**, 1337-1342 (2001).

41 Kobayashi, A., Kobayashi, Y. & Shiomi, K. Fish allergy in patients with parvalbumin specific immunoglobulin E depends on parvalbumin content rather than molecular differences in the protein among fish species. *Biosci. Biotech. Bioch.* **80**, 2018-2021 (2016).

42 Sharp, M. F. & Lopata, A. L. Fish Allergy: in Review. *Clin. Rev. Allerg. Immu.* **46**, 258-271 (2014).

43 Smargiassi, M. *et al.* Chemical basis of prey recognition in Thamnophiine snakes: The unexpected new roles of parvalbumins. *PloS One* **7**, e39560 (2012).

44 Arif, S. H. A Ca2+ binding protein with numerous roles and uses: parvalbumin in molecular biology and physiology. *BioEssays* **31**, 410-421 (2009).

45 Patz, S., Grabert, J., Gorba, T., Wirth, M. J. & Wahle, P. Parvalbumin expression in visual cortical interneurons depends on neuronal activity and TrkB ligands during an early period of postnatal development. *Cereb. Cortex* **14**, 342-351 (2004).

46 Basu, N. *et al.* Heat shock protein genes and their functional significance in fish. *Gene* **295**, 173-183 (2002).

47 Deffit, S. N. & Blum, J. S. A central role for HSC70 in regulating antigen trafficking and MHC class II presentation. *Mol. Immunol.* **68**, 85-88 (2015).

48 Stricher, F., Macri, C., Ruff, M. & Muller, S. HSPA8/HSC70 chaperone protein: structure, function and chemical targeting. *Autophagy* **9**, 1937-1954 (2013).

49 Krasnov, A., Skugor, S., Todorcevic, M., Glover, K. A. & Nilsen, F. Gene expression in Atlantic salmon skin in response to infection with the parasitic copepod *Lepeophtheirus salmonis*, cortisol implant, and their combination. *BMC Genomics* **13**, 130 (2012).

50 Mohindra, V., Tripathi, R. K., Yadav, P., Singh, R. K. & Lal, K. K. Hypoxia induced altered expression of heat shock protein genes (Hsc71, Hsp90α and Hsp10) in Indian catfish, *Clarias batrachus* (Linnaeus, 1758) under oxidative stress. *Mol. Biol. Rep.* **42**, 1197-1209 (2015).

51 McDonough, H. & Patterson, C. CHIP: a link between the chaperone and proteasome systems. *Cell Stress Chaperon.* **8**, 303-308 (2003).

52 Xu, L. *et al.* Proteomic responses reveal the differential effects induced by cadmium in mussels *Mytilus galloprovincialis* at early life stages. *Fish Shellfish Immunol.* **55**, 510-515, doi:http://dx.doi.org/10.1016/j.fsi.2016.06.017 (2016).

53 Houston, D. S., Carson, C. W. & Esmon, C. T. Endothelial cells and extracellular calmodulin inhibit monocyte tumor necrosis factor release and augment neutrophil elastase release. *J. Biol. Chem.* **272**, 11778-11785 (1997).

54 Li, S., Jia, Z., Li, X., Geng, X. & Sun, J. Calmodulin is a stress and immune response gene in Chinese mitten crab *Eriocheir sinensis*. *Fish Shellfish Immunol.* **40**, 120-128 (2014).

55 Lockwood, B. L. & Somero, G. N. Transcriptomic responses to salinity stress in invasive and native blue mussels (genus *Mytilus*). *Mol. Ecol.* **20**, 517-529 (2011).

56 Concha, M. I., Molina, S. a., Oyarzún, C., Villanueva, J. & Amthauer, R. Local expression of apolipoprotein A1 gene and a possible role for HDL in primary defence in the carp skin. *Fish Shellfish Immunol.* **14**, 259-273 (2003).

57 Valdenegro-Vega, V. A. *et al.* Differentially expressed proteins in gill and skin mucus of Atlantic salmon (*Salmo salar*) affected by amoebic gill disease. *Fish Shellfish Immunol.* **40**, 69-77 (2014).

58 Stafford, J. L., Neumann, N. F. & Belosevic, M. Products of proteolytic cleavage of transferrin induce nitric oxide response of goldfish macrophages. *Dev. Comp. Immunol.* **25**, 101-115 (2001).

59 Trites, M. J. & Barreda, D. R. Contributions of transferrin to acute inflammation in the goldfish, *C. auratus*. *Dev. Comp. Immunol.* **67**, 300-309 (2017).

60 Valero, Y., Martínez-Morcillo, J. F., Esteban, Á. M., Chaves-Pozo, E. & Cuesta, A. Fish peroxiredoxins and their role in immunity. *Biology* **4**, doi:10.3390/biology4040860 (2015).

61 Forné, I., Abián, J. & Cerdà, J. Fish proteome analysis: Model organisms and non-sequenced species. *Proteomics* **10**, 858-872, doi:10.1002/pmic.200900609 (2010).

62 Rajan, B., Kiron, V., Fernandes, J. M. O. & Brinchmann, M. F. Localization and functional properties of two galectin-1 proteins in Atlantic cod (*Gadus morhua*) mucosal tissues. *Dev. Comp. Immunol.* **40**, 83-93 (2013).

63 Gygi, S. P., Rochon, Y., Franza, B. R. & Aebersold, R. Correlation between protein and mRNA abundance in yeast. *Mol. Cell Biol.* **19**, 1720-1730 (1999).

64 Maier, T., Güell, M. & Serrano, L. Correlation of mRNA and protein in complex biological samples. *FEBS Lett.* **583**, 3966-3973 (2009).

65 Cordero, H., Ceballos-Francisco, D., Cuesta, A. & Esteban, M. Á. Dorso-ventral skin characterization of the farmed fish gilthead seabream (*Sparus aurata*). *PloS One* **12**, e0180438 (2017).

66 Elliott, D. in *The laboratory fish* (ed Otrander GK) Ch. 17, 271-306 (Academic press, 2000).

67 Djurdjevič, I., Kreft, M. E. & Sušnik Bajec, S. Comparison of pigment cell ultrastructure and organisation in the dermis of marble trout and brown trout, and first description of erythrophore ultrastructure in salmonids. *J. Anat.* **227**, 583-595 (2015).

68 Ottesen, O. H. & Olafsen, J. A. Ontogenetic development and composition of the mucous cells and the occurrence of saccular cells in the epidermis of Atlantic halibut. *J. Fish Biol.* **50**, 620-633 (1997).

69 Mittal, A. K., Ueda, T., Fujimori, O. & Yamada, K. Histochemical analysis of glycoproteins in the unicellular glands in the epidermis of an Indian freshwater fish *Mastacembelus pancalus* (Hamilton). *Histochem. J.* **26**, 666-677 (1994).

70 Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* **29**, e45-e45 (2001).

71 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Meth.* **9**, 676-682 (2012).

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Author Contributions
MFB and MHI conceived the study. DMP sampled the tissues, performed the experiments and analysis. DMP and MB designed and performed the mRNA expression study. DMP and KB conducted the DNA sequencing. DMP and MFB wrote the manuscript. All authors reviewed the manuscript.

Additional Information
The authors declare no competing financial interests.

Figure legends

Fig 1. **Lumpsucker image indicating different regions of skin sampled in this study.** Skin from three regions was sampled for proteomics, mRNA expression and histology in this study. D; dorsal region above the lateral line near operculum, C; caudal region below the lateral line near caudal peduncle, V; ventral region near the adhesive disc.

Fig 2. **A 12.5% acrylamide gel image showing spots that were excised and sequenced.** Spots circled in yellow are identified but not differentially expressed, spots circled in red are identified and differentially expressed, spots in yellow rectangles are not identified neither differentially expressed, spots in red rectangles are not identified but differentially expressed. Protein identities are found in Table 1.

Fig 3. **Differentially expressed skin proteins shown as spot intensities in bar graphs and spots in 12.5% acrylamide gels a)** Spot intensities (X-axis) of differentially expressed proteins among dorsal (D), caudal (C) and ventral (V) region of lumpsucker skin based on PD Quest analysis. One way ANOVA and Tukey HSD post hoc analysis were used for normally distributed data whereas Kruskal-Wallis test and Dunn's test were used for non parametric data. Error bar shows the error of mean. Bars with different letters are significantly different, $p<0.05$, $n = 6$. **b)** Gel images from dorsal, caudal and ventral regions showing the differentially expressed proteins. Spots are encircled in red and assigned by specific spot numbers.

Fig 4. **Protein interaction map of identified lumpsucker skin proteins.** A possible protein-protein interaction map with high edge confidence was generated by string v.10. Thicker edges (line joining the nodes) represent a confidence of 0.900/1. slightly thinner edges represent a confidence of 0.700/1. Edges represent protein-protein association where association does not necessarily mean physical binding of the proteins, there could be involvement of several proteins to a shared function. Protein interaction network is created using zebrafish orthologues of the proteins identified in lumpsucker skin. Full

protein name for the abbreviation are provided as Supplementary Table S1. Role of these proteins are mentioned in results and discussion sections.

Fig 5. **mRNA expression level of few immune related genes in teleosts.** mRNA expression level of selected genes between the three different regions (D, C, V). The expression is relative to the geometric mean of three reference genes *ef1-alfa* (elongation factor alfa1), *β-actin* and *gapdh* (glyceraldehyde 3-phosphate dehydrogenase). Target genes in X-axis are *apoa1* (apolipoprotein A1), *calm* (calmodulin), *hspa8* (heat shock cognate 71 kDa), *hist1h2b* (histone h2b)

Fig 6. **Lumpsucker skin sections showing structural differences among dorsal, caudal and ventral regions of skin**. Representative photomicrographs of dorsal **(a)**, caudal **(b)** and ventral **(c)** regions of lumpsucker skin sections stained with periodic acid Schiff and alcian blue. The letters represent different cells and layers in skin. a) epithelial layer, b) epidermal squamous epithelial cells, c) goblet cells, d) saccular cells e) dermal pigment deposition f) basal layer g) dermis. Differences in goblet cell counts **(d)** and epidermal thickness **(e)** between the three skin regions D (dorsal), C (caudal), V (ventral) are shown. Error bars represent mean ± SE, n=6, $p<0.05$.

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Table 1. Lumpsucker skin proteins identified by MASCOT

Spot ID	Protein name (Species)	Accession number	Apparent PI/MW	Significant threshold/ p-value	Protein score/Up	SC (%)	Peptide sequence	GO terms	Data base
D2	Prefoldin subunit 1 (*Danio rerio*)	Q5D016; A7MCD8	8.83/13978	21/p<0.05	45/45	9	KLADLQIEQLSRV	protein folding (GO:0006457)	Swiss prot
D3	Glial fibrillary acidic protein (*Carassius auratus*)	P48677	4.93/42636	20/ p<0.05	28/28	3	KLALDIEIATYRK	cytoskeleton organization (GO:0007010)	Swiss prot
D5	40S ribosomal protein S12 (*Oreochromis niloticus*)	O13019	6.30/14726	20/ p<0.045	159/159	24	KDVIEEYFKS KLGEWVGLCKI KLVEALCAEHQINLIKV	rRNA processing (GO:0006364)	Swiss prot
D6	Glial fibrillary acidic protein (*Carassius auratus*)	P48677	4.93/42636	20/ p<0.05	41/41	3	KLALDIEIATYRK	cytoskeleton organization (GO:0007010)	Swiss Prot
D8	Fatty acid binding protein (*Salvelinus fontinalis*)	AIK01712	5.77/8299	49/ p<0.037	62/62	10	LGVGFATRQ	epidermis development (GO:0008544)	NCBI
D9	Ubiquitin-40S ribosomal protein (*Ictalurus punctatus*)	P68200; P68199; Q90YP4	9.68/18330	20/ p<0.049	135/135	17	KEGIPPDQQRL KESTLHLVLRL RTLSDYNIQKE	nucleotide excision repair, DNA damage recognition (GO:0000715)	Swiss Prot
D10	Hemoglobin subunit beta (*Cottoperca gobio*)	P84652	6.96/16481	20/ p<0.05	50/50	12	MVEWTDFERA KFLAVVVSSLGRQ	transport (GO:0006810)	Swiss prot
D11	Hemoglobin subunit beta-2 (*Pseudaphritis urvillii*)	P83625	5.93/16443	21/ p<0.045	176/138	26	RATIKDIFSKI VEWTDFERA KFLAVVVSSLGRQ RCLVVYPWTQRY	transport (GO:0006810)	Swiss Prot
D12	Parvalbumin beta-2 (*Theragra chalcogramma*)	Q90YK7	4.60/11614	22/ p<0.035	47/47	17	KLFLQNFSASARA	calcium ion binding (GO:0005509)	Swiss Prot
D13	Lipocalin-like (*Xiphophorus maculatus*)	XP_00580 3374	4.94/21354	50/ p<0.03	118/118	5	KDGVSEVLNKL KTKDGVSEVLNKL	Lipid metabolic process (GO:0006629)	NCBI
D14	Collagen alpha1 (I) chain like isoform X1 (*Hippocamus comes*)	XP_01974 0420	5.72/138015	48/p<0.05	129/129	1	RTGGSCTLDGQVFADRD	skeletal system development (GO:0001501)	NCBI
D15	Natterin-2 (*Thalassophryne nattereri*)	Q66S21	8.90/41985	21/ p<0.049	28/28	2	KADIPFTATLIRT	NA	Swiss Prot
D16	Keratin, type II cytoskeletal 8 (*Danio rerio*)	Q6NWF6; Q7ZT78	5.15/57780	23/ p<0.03	39/39	1	RFASFIDKV	keratinization (GO:0031424), extrinsic	Swiss Prot

								apoptotic signaling pathway (GO:0097191)	
D17	Collagen alpha-2(I) chain isoform X1 (*Stegastes partitus*)	XP_008287498	9.41/127388	51/p<0.02	535/154	4	RAKDYEVDATIK.S KAVVLQGSNDVELRA RFTFSVLEDGCTRH KKAVVLQGSNDVELRA RLPLLDIAPLDIGGADQE FGLDIGPVCFK	skeletal system development (GO:0001501)	Swiss Prot
D18	Calmodulin (*Ctenopharyngodon idella*)	Q6IT78	4.09/16827	25/p<0.019	25/25	10	KELGTVMRS KDTDSEEEIRE	calcium ion binding (GO:0005509)	Swiss Prot
D20	60S ribosomal protein L30 (*Ictalurus punctatus*)	P58372	9.65/13105	20/p<0.05	91/91	24	KLVILANNCPALRK RVCTLAIIDPGDSDIIRS	antimicrobial humoral immune response mediated by antimicrobial peptides (GO:0061844)	Swiss Prot
D21	Histone H2A (*Danio rerio)*	Q71PD7	10.58/13501	20/p<0.05	55/55	14	RAGLQFPVGRI RGDEELDSLIKA	cellular response to DNA damage stimulus (GO:0006974)	Swiss Prot
D22	Fatty acid-binding protein (*Maylandia zebra*)	XP_004549259	6.34/15269	49/p<0.04	208/133	23	KAIGVGFATRQ KCIMGDVIAVRT KLNEPFDETTADDRK	epidermis development (GO:0008544)	NCBI
D23	Histone H2B (*Danio rerio*)	Q5BJA5	10.37/13569	20/p<0.05	64/64	12	REIQTAVRL RLLLPGELAKH	innate immune response in mucosa (GO:0002227)	Swiss Prot
D24	Histone H4 (*Oncorhynchus mykiss*)	P62797; P02304; P02305	11.36/11360	21/p<0.05	24/24	7	KVFLENVIRD	nucleosome assembly (GO:0006334)	Swiss Prot
D25	Parvalbumin beta (*Scomber japonicus*)	P59747; Q7ZW61	5.15/11652	22/p<0.035	52/52	16	KSGFIEEEELKL	calcium ion binding (GO:0005509)	Swiss Prot
D26	Myotrophin (*Takifugu rubripes*)	XP_003972762	4.96/12996	52/p<0.05	53/53	7	KLVTAEDVNRT	cellular response to mechanical stimulus (GO:0071260)	NCBI
D27	unnamed protein product (Tetraodon nigroviridis)	CAG11620	5.96/18450	59/p<0.0035	60/60	7	KALAAGGVGSIVRV	NA	NCBI
D28	Ubiquitin-conjugating enzyme E2 (*Danio rerio*)	Q6PEH5	7.79/16370	28/p<0.0047	68/68	6	RLLEELEEGQKG	cellular response to DNA damage stimulus (GO:0006974)	Swiss Prot
D29	Myosin light chain 2, isoform	CAD3255	4.72/19213	54/p<0.013	154/154	14	KGADPEDVIITAFKV	muscle contraction	NCBI

	B (*Hippoglossus hippoglossus*)	2					KVLDPEATGSIKK	(GO:0006936)	
D30	Apolipoprotein A1 (*Xiphophorus maculatus*)	XP_005799476	4.68/28589	49/p<0.045	122/122	7	KVQVELTQRA KDLQAQLGPYTDDLKQ	lipoprotein metabolic process (GO:0042157)	NCBI
D31	Apolipoprotein A1, partial (*Morone saxatilis*)	ACH90227	4.75/20591	58/p<0.004	94/94	7	KALDQLDDTEYKELKA	lipoprotein metabolic process (GO:0042157)	NCBI
D32	Apolipoprotein A1, partial (*Morone saxatilis*)	ACH90227	4.75/20591	53/p<0.015	78/78	6	KALDQLDDTEYKE	lipoprotein metabolic process (GO:0042157)	NCBI
D33	Protein AMBP (*Pleuronectes platessa*)	P36992	5.30/40499	24/p<0.05	45/45	2	RDTVLDDFKT	cell adhesion (GO:0007155)	Swiss Prot
D34	40S ribosomal protein S3 (*Ictalurus punctatus*)	90YS2	9.8/27044	20/p<0.04	41/41	3	RTEIIILATRT	nuclear transcribed mRNA metabolic process (GO:0000184)	Swiss Prot
D35	Collagen alpha-2(I) chain *(Oncorhynchus mykiss*)	_	9.36/127364	21/p<0.035	249/249	1	KAVLLQGSNDVELRA KKAVLLQGSNDVELRA	skeletal system development(GO:0001501)	Swiss Prot
D36	Actin, cytoplasmic 1 (*Ctenopharyngodon idella*)	83751; O73815; P12714	5.30/42068	30/p<0.005	47/47	1	KIIAPPERK	ATP dependent chromation remodelling (GO:0043044)	Swiss Prot
D37	Transcriptional activator protein Pur-beta (*Danio rerio*)	Q6PHK6; Q6NW99	5.53/32586	20/p<0.05	34/34	3	KIAEVGAGGSKS	transcription (GO:0006351)	
D38	Actin, alpha cardiac (*Takifugu rubripes)*	P53480	5.22/42290	20/p<0.05	988/381	54	KIIAPPERK KRGILTLKY KAGFAGDDAPRA RDLTDYLMKI RGYSFVTTAERE KEITALAPSTMKI KDSYVGDEAQSKR RAVFPSIVGRPRH RHQGVMVGMGQKD KQEYDEAGPSIVHRK KIWHHTFYNELRV KSYELPDGQVITIGNERF RVAPEEHPTLLTEAPLNPKA KYPIEHGIITNWDDMEKI	actin filament based movement (GO:0030048), apoptotic process (GO:0006915)	Swiss Prot

							RKDLYANNVLSGGTTM YPGIADRM KLCYVALDFENEMATA ASSSSLEKS		
D39	Hemoglobin subunit beta-1 (*Liparis tunicatus*)	P85082	6.64/16660	25/p<0.022	212/176	23	KLHVDPDNFKL KAFTGEVQAALQKF KLISDCLTIVVASRL	transport (GO:0006810)	Swiss Prot
D40	Hemoglobin subunit beta-1 (*Liparis tunicatus*)	P85082	6.64/16660	20/p<0.05	81/81	17	KAFTGEVQAALQKF KLISDCLTIVVASRL	transport (GO:0006810)	Swiss Prot
D41	Actin cytoplasmic 1 (*Ctenopharyngodon idella*)	P83751; O73815; P12714	5.30/42068	20/p<0.05	76/54	9	KDSYVGDEAQSKR KLCYVALDFEQEMGTA ASSSSLEKS	ATP dependent chromation remodelling (GO:0043044)	Swiss Prot
D42	Heat shock cognate 71 kDa (*Oryzias latipes*)	Q9W6Y1	5.80/76577	24/p<0.02	188/112	5	R.GTLDPVEK.S R.TTPSYVAFTDTER.L R.IINEPTAAAIAYGLDK. K RIINEPTAAAIAYGLDKK V	cellular response to stress (GO:1900034, GO:0009267), chaperone activity (GO:0061684, GO:0051085)	Swiss Prot
D43	Transferrin (*Trachidermus fasciatus*)	AEV21971	5.80/75092	49/p<0.03	438/130	7	KANYELLCKD KHLTVPESEKA KQTGDCDFTKF KSSGLTWETLKG KEADAMAVDGGQVYTA GKC RKEADAMAVDGGQVYT AGKC	cellular iron ion homeostasis (GO:0006879)	NCBI
D44	Serotransferrin (*Paralichthys olivaceus*)	O93429	6.06/76545	20/p<0.05	59/59	2	KEADAMAVDGGQVYTA GKC	cellular iron ion homeostasis (GO:0006879)	Swiss Prot
D45	Triosephosphate isomerase B (*Danio rerio*)	Q90XG0; Q7T315	6.45/27096	28/p<0.015	261/261	25	KFFVGGNWKM KGAFTGEISPAMIKD KTASPQQAQEVHDKL RHVFGESDELIGQKV KVVLAYEPVWAIGTGKT	glucose metabolic process (GO:0006006)	Swiss Prot
D46	Adenylate kinase isoenzyme 1 (*Cyprinus carpio*)	P12115	6.64/21532	21/p<0.05	171/171	26	RSDDNEETIKK KGYLIDGYPRE KATEPVIAYYETRG KQLQAIMQKG	ATP metabolic process (GO:0046034)	Swiss Prot

							KGELVPLDTVLDMIKD		
D47	Protein disulfide-isomerase precursor (*Ictalurus punctatus*)	JZ585147	5.17/31098	57/p<0.04	252/72	18	KSNQLPLVIEFTEQTAPKI KSNQLPLVIEFTEQTAPKI KGKILFIFIDSDVDDNQRI	cell redox homeostasis (GO:0045454)	NCBI
D48	Protein disulfide-isomerase (*Maylandia zebra*)	XP_004538825	4.57/57378	47/p<0.05	77/77	3	KVDATEETELAQEYGVRG	cell redox homeostasis (GO:0045454)	NCBI
D50	Cofilin-1 (*Onchorynchus mykiss*)	ACO07669	8.48/18784	50/p<0.03	87/87	6	RYALYDATYETKE	actin cytoskeleton organization (GO:0030036)	NCBI
D52	Transcription factor BTF3 homolog 4 (*Danio rerio*)	Q6PC91	5.95/17350	21/p<0.05	29/29	5	KLAEQFPRQ	NA	Swiss Prot
D55	40S ribosomal protein S25 (*Danio rerio*)	Q6PBI5	10.14/13852	20/p<0.05	109/109	16	KLITPAVVSERL RDKLNNLVLFDKA	nuclear transcribed mRNA metabolic process (GO:0000184)	Swiss Prot
D56	ATP synthase subunit d, mitochondrial-like (*Lates calcarifer*)	XP_018535231	7.85/18181	48/p<0.05	118/118	11	KSASAYIEGSKA KAGMVDEFEKK	ATP biosynthetic process (GO:0006754)	NCBI
D57	Proteasome subunit alpha type-5, partial (*Larimichthys crocea*)	KKF13888	4.77/25745	48/p<0.054	419/419	29	RGVNTFSPEGRL RLFQVEYAIEAIKL RITSPLMEPNSIEKI KLNATNIELATVEPGKT RAIGSASEGAQSSLQEV YHKS	antigen processing and presentation of exogenous peptide antigen via MHC class I (GO:0002479)	NCBI
D59	Cofilin-2 (*Onchorynchus mykiss*)	ACO07669	8.84/18784	48/p<0.05	83/83	6	RYALYDATYETKE	actin filament depolymerisation (GO:0030042)	NCBI
D60	Unnamed protein product, partial (*Tetraodon nigroviridis*)	CAG09787	6.82/18856	52/p<0.025	146/146	14	RYALYDATYETKE KVTDEVIAVFNDMKV	NA	NCBI
D62	60S acidic ribosomal protein P0 (*Danio rerio*)	Q9PV90	6.16/34902	21/p<0.05	40/40	6	RGNVGFVFTKE	translation (GO:0006412)	Swiss Prot
D63	Guanine nucleotide-binding protein subunit beta-2-like 1 (*Oreochromis niloticus*)	O42249	8.07/35541	28/p<0.01	570/570	35	KIIVDELRQ KIWDLEGKI RQEVISTNSKA RVWQVTIGTR KLWNTLGVCKY	activation of adenylate kinase activity (GO:0007190), cellular response to catecholamine stimulus (GO:0071870)	Swiss Prot

							RDETNYGIPQRS KDVLSVAFSADNRQ RYWLCAATGPSIKI KDGQAMLWDLNEGKH KGHSGWVTQIATTPKYP DMILSASRD		
D64	Guanine nucleotide-binding protein subunit beta-2-like 1 (*Oreochromis niloticus*)	O42249	8.07/35541	20/p<0.025	381/381	37	KIIVDELRQ KIWDLEGKI RVWQVTIGTR RDETNYGIPQRS KDVLSVAFSADNRQ RYWLCAATGPSIKI KDGQAMLWDLNEGKH KIIVDELRQEVISTNSKA	activation of adenylate kinase activity (GO:0007190), cellular response to catecholamine stimulus (GO:0071870)	Swiss Prot
D65 D66	60S acidic ribosomal protein P0 (*Danio rerio*)	Q9PV90	6.16/34902	21/p<0.05	108/108	6	RGNVGFVFTKE KTSFFQALGITTKI	translation (GO:0006412)	Swiss Prot
D67	costars family protein ABRACL (*Larimichthys crocea*)	XP_01073 7667	5.66/9091	56/p<0.007	55/55	9	KLLVEEIQRL	NA	NCBI
D68	Parvalbumin-2 (*Danio rerio*)	Q9I8V0; Q567L1	4.46/11672	20/p<0.05	147/147	20	KIGVDEFALLVKA KLFLQNFSAGARA	regulation of calcium ion concentration (GO:0051480)	Swiss Prot
D69 D70	Elongation factor 1-alpha (*Danio rerio*)	Q92005	9.16/50301	21/p<0.04	47/47	2	KIGGIGTVPVGRV	transcription (GO:0006351), cellular response to epidermal growth factor stimulus (GO:0071364)	Swiss Prot
D71	ATP synthase subunit beta, mitochondrial (*Cyprinus carpio*)	Q9PTY0	5.05/55327	28/p<0.01	709/709	28	KVLDTGAPIRI KIGLFGGAGVGKT RIPVGPETLGRI KVVDLLAPYAKG RTIAMDGTEGLVRG RIMNVIGEPIDERG RFTQAGSEVSALLGRI KTVLIMELINNVAKA K.VALVYGQMNEPPGAR .A	ATP biosynthetic process (GO:0006754)	Swiss Prot

							RDQEGQDVLLFIDNIFRF REGNDLYHEMIESGVIN LKD KSLQDIIAILGMDELSEE DKLTVARA		
D72	Tubulin beta-1 chain (*Gadus morhua*)	Q9YHC3	4.79/50173	31/0.004	1063/212	35	KTAVCDIPPRG RYLTVAAIFRG KNMMAACDPRH RFPGQLNADLRK KLAVNMVPFPRL RISEQFTAMFRR RKLAVNMVPFPRL RINVYYNEASGGKY RIMNTFSVVPSPKV KEVDEQMLNVQNKN RLHFFMPGFAPLTSRG REIVHLQAGQCGNQIGA KF KMAATFIGNSTAIQELFK R KGHYTEGAELVDSVLD VVRK	spindle assembly (GO:0051225)	Swiss Prot
D73	Keratin, type II cytoskeletal 8 (*Danio rerio*)	Q6NWF6; Q7ZT78;	5.15/57780	30/p<0.006	333/333	8	RFASFIDKV RFLEQQNKM KLLEGEEDRL KYEDEINKRT RSNIDAMFEAYIANLRR	keratinization (GO:0031424), extrinsic apoptotic signaling pathway (GO:0097191)	Swiss Prot
D74	Keratin, type II cytoskeletal 8 (*Danio rerio*)	Q6NWF6; Q7ZT78	5.15/57780	27/p<0.008	306/306	11	RFASFIDKV RFLEQQNKM KLLEGEEDRL RAQYEDIANRS KLESLTDEINFLRQ KLEADLHNMQGLVEDF KN	keratinization (GO:0031424), extrinsic apoptotic signaling pathway (GO:0097191)	Swiss Prot
D75	Glyceraldehyde-3-phosphate dehydrogenase 2 (*Danio rerio*)	Q5MJ86	6.55/36426	22/p<0.03	167/167	12	RGAHQNIIPASTGAAKA RVPVADVSVVDLTCRL	antimicrobial humoral immune response mediated by antimicrobial peptide	Swiss Prot

								(GO:0061844)	
D76	Protein AMBP (*Pleuronectes platessa*)	P36992	5.30/40499	20/p<0.05	40/40	2	RDTVLDDFKT	cell adhesion (GO:0007155)	Swiss Prot
D78	60S ribosomal protein L18 (*Salmo salar*)	P24558	11.94/20563	21/p<0.05	25/25	4	KSVLLSAPRN	rRNA processing (GO:0006364)	Swiss Prot
D80	Rhophilin-2 (*Danio rerio*)	Q6TNR1; Q803B1	7.55/77692	20/p<0.05	35/35	1	KAEMEIPAATKV	signal transduction (GO:0007165)	Swiss Prot
D82	Tropomyosin alpha-1 chain (*Danio rerio*)	P13104	4.70/32760	25/p<0.02	246/246	16	KLDKENALDRA RIQLVEEELDRA KTIDDLEDELYAQKL KAISEELDHALNDMTSI	actin filament organization (GO:0007015)	Swiss Prot
D83	40S ribosomal protein SA (*Danio rerio*)	Q803F6	4.75/34162	23/p<0.03	232/94	19	RLLIVTDPRA RFTPGTFTNQIQAAFRE RAIVAIENPADVCVISSR N REHPWEVMPDLYFYRD PEEIEKE	cell adhesion (GO:0007155)	Swiss Prot
D84	Malate dehydrogenase 2-2, NAD (mitochondrial) (*Oreochromis niloticus*)	CDQ84176	8.15/35782	53/p<0.015	508/180	30	KYFSTPLLLGKH RVFGVTTLDIVRA RFTFSVLDAMNGKE KAGAGSATLSMAYAGA RF KVEFPADQLSALTARI KVAVLGASGGIGQPLSL LLKN RDDLFNTNATIVATLAD AVARN	carbohydrate metabolic process (GO:0005975)	NCBI
D85	Annexin A1 (*Notothenia coriiceps*)	XP_010788362	6.50/37631	48/p<0.05	79/79	5	KGDLEEVVLALLKT	adaptive immune response (GO:0002250)	NCBI
D86	N-acylneuraminate cytidylyltransferase B (*Danio rerio*)	H9BFW7; E7F0X7	6.30/48034	21/p<0.05	22/22	2	RAALILARGGSKG	metabolic process (GO:0006054)	Swiss Prot
D88	Proteasome subunit alpha type-2 (*Carassius auratus*)	O73672	5.99/25918	21/p<0.05	120/120	15	KASNGVVLATEKK RGYSFSLTTFSPSGKL	antigen processing and presentation of exogenous peptide antigen via MHC class I (GO:0002479)	Swiss Prot
D89	Triosephosphate isomerase A	Q1MTI4;	4.90/27179	20/p<0.05	38/38	5	RHVFGESDELIGQKV	glucose metabolic process	Swiss

	(*Danio rerio*)	Q7ZWB0; Q90XF9						(GO:0006006)	Prot
D91	COP9 signalosome complex subunit 2 (*Danio rerio*)	Q6IQT4	5.31/51824	24/p<0.025	28/28	1	KQMIKINFKL	negative regulation of transcription(GO:0000122)	Swiss Prot
D92	Elongation factor 1-delta (*Salmo salar*)	ACM08644	4.59/26363	48/p<0.05	58/58	7	KSSILLDVKPWDDETDMSKL	transcription (GO:0006351)	NCBI
D93	Myosin light chain 1, skeletal muscle isoform (*Liza ramada*)	P82159	4.54/20054	21/ p<0.05	89/89	11	RIVLSTLGEKM KEGNGTVMGAELRI	muscle contraction (GO:0006936)	Swiss Prot
D95	Collagen alpha-2(I) chain (*Larimichthys crocea*)	KKF26459	9.15/119089	55/p<0.0087	142/142	2	RFTFSVLEDGCTRH KSLNTQIENLLTPEGSRK	skeletal system development(GO:0001501)	NCBI
D96	Natural killer enhancing factor (*Scopthalmus maximus*)	ABF01135	5.58/22063	48/p<0.048	181/181	17	KIPIVADLTKT RQITINDLPVGRS RDYGVLKEDDGIAYRG	response to reactive oxygen species (GO:0000302)	NCBI
D97	Proteasome subunit beta type 6 (*Osmerus mordax*)	ACO09878	5.13/25238	52/p<0.02	64/64	4	RTTTGAYIANRV	antigen processing and presentation of exogenous peptide antigen via MHC class I (GO:0002479)	NCBI

PI; isoelectric point, MW; molecular weight, Up; total score of unique peptide, SC; sequence coverage, NA; data not available, GO terms; gene ontology term of biological process of the proteins retrieved manually from UniProt KB, Missing spot number signifies that those proteins are not identified. Unique peptides are marked in red letters and peptides in black letters are not unique.

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Table 2. Oligonucleotide sequences used in the study

Gene name	Primer sequence	Amplicon size	Primer efficiency (%)	Purpose of use
apoA1	F: TACMTRRCTCRGGTGAARGASA R: CTTGTAYTCYKSARCRTAGGG	528	-	Degenerate PCR
	F: ACATGCACACCAAGCTCAG R: AATGATTGAGGAGCGGAAG	107	98.678	qPCR
calm	F: CAGATTGCHGARTTYAARGARGC R: GTTGACCTGDCCRTCWCCRT	392	-	Degenerate PCR
	F: ACGGACAGTGAGGAGGAGA R: TTCTCCCCGAGGTTAGTCA	110	87.770	qPCR
hspa8	F: GGCACTACCTACTCCTGTGTAG R: TTTGAACTCRGAGATGAAGTGG	706	-	Degenerate PCR
	F: TCTCATTGGACGTCGGTTT R: TGGTCTCGCCCTTGTACTC	119	101.439	qPCR
hist1h2b	F: ACCAGGAAGGAGAGCTATGCYATC R: CTTGGTGACGGCCTTKGTDC	268	-	Degenerate PCR
	F: ATCTTTGAGCGCATCGCCG R: TGTTCCCTCAGACACCGCG	144	104.115	qPCR
gapdh	F: GCCATCAAYGACCCMTTCAT R: GCAGTTRGTVGTGCAGGADG	380	-	Degenerate PCR
	F: GGGGCAAGCTCATCGTCG R: CCTGGATGTGAGAGGAGGCC	149	104.03	qPCR
β-actin	F: GACTACCTCATGAAGATCCTGA R: GGTGATGACCTGTCCGTC	188		qPCR
ef1-alfa	F: AAGTTCGAGAAGGAAGCCGC R: ATGGTGATACCACGCTCACG	98	100.584	qPCR

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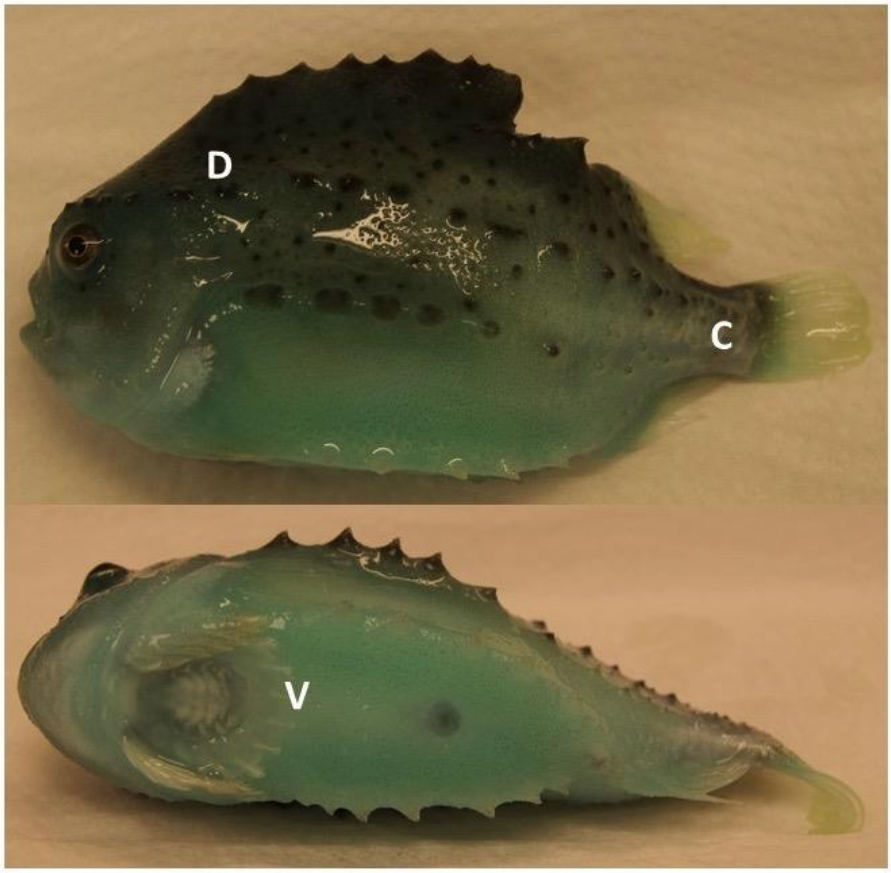
Fig 1



699
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Fig 2

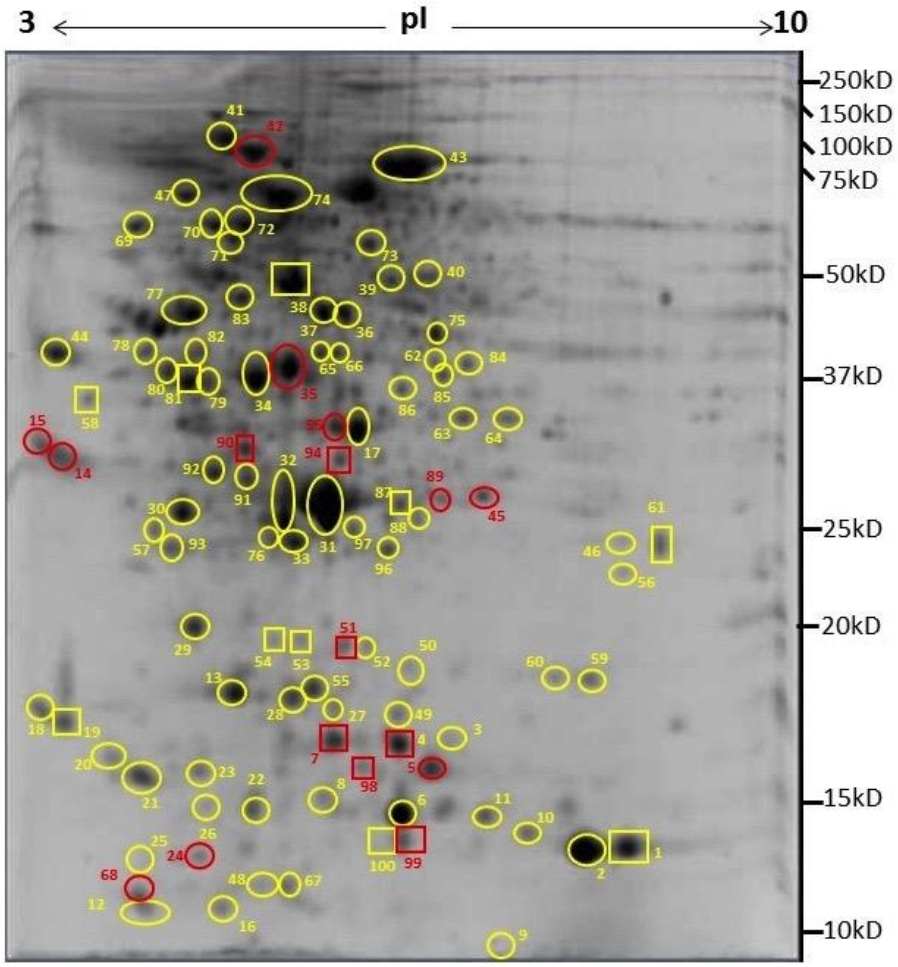

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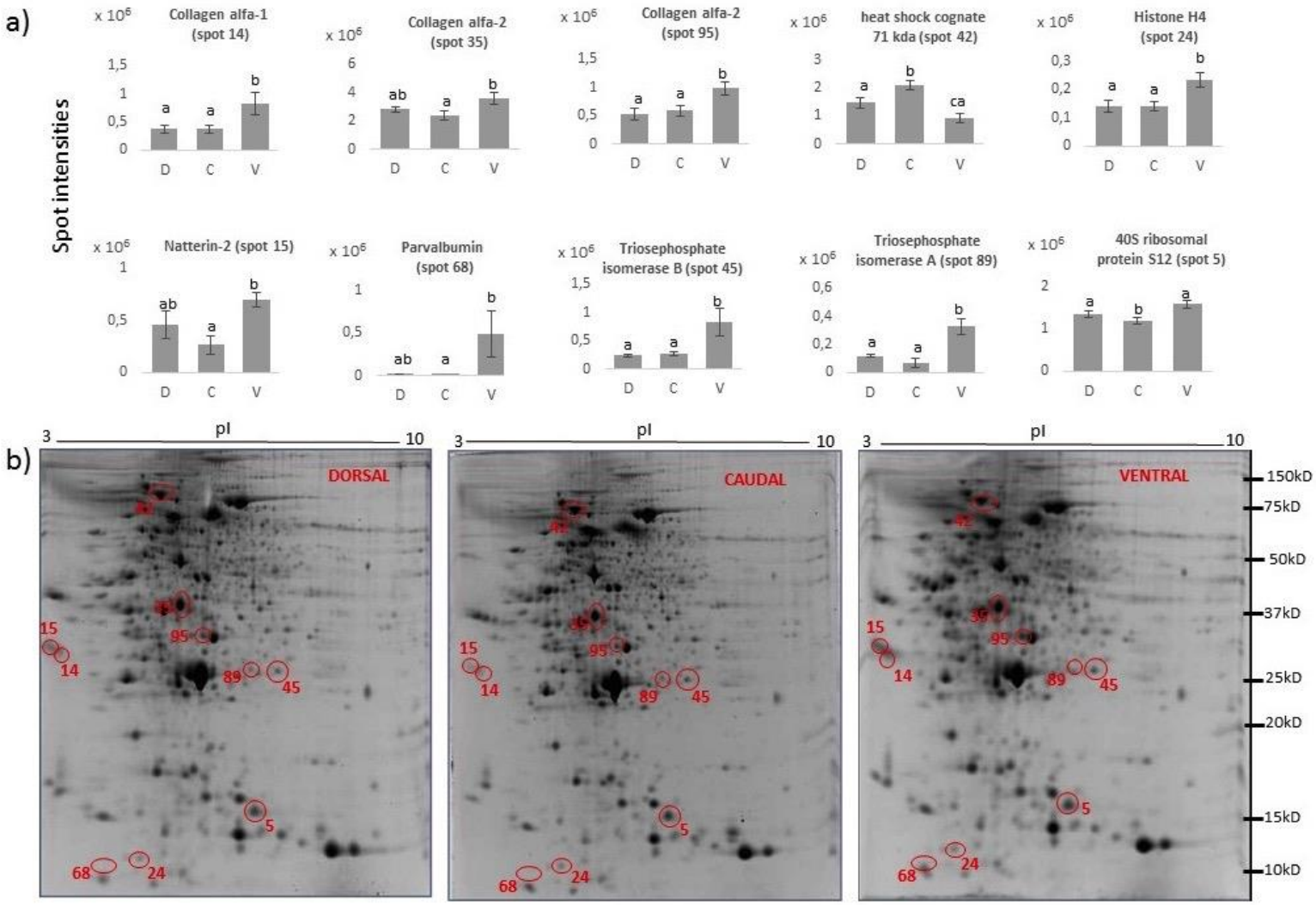
Fig 3


a)
Spot intensities
x 10⁶
Collagen alfa-1 (spot 14)
Collagen alfa-2 (spot 35)
Collagen alfa-2 (spot 95)
heat shock cognate 71 kda (spot 42)
Histone H4 (spot 24)
Natterin-2 (spot 15)
Parvalbumin (spot 68)
Triosephosphate isomerase B (spot 45)
Triosephosphate isomerase A (spot 89)
40S ribosomal protein S12 (spot 5)
D
C
V
b)
pI
3
10
DORSAL
CAUDAL
VENTRAL
150kD
75kD
50kD
37kD
25kD
20kD
15kD
10kD

706

Fig 4

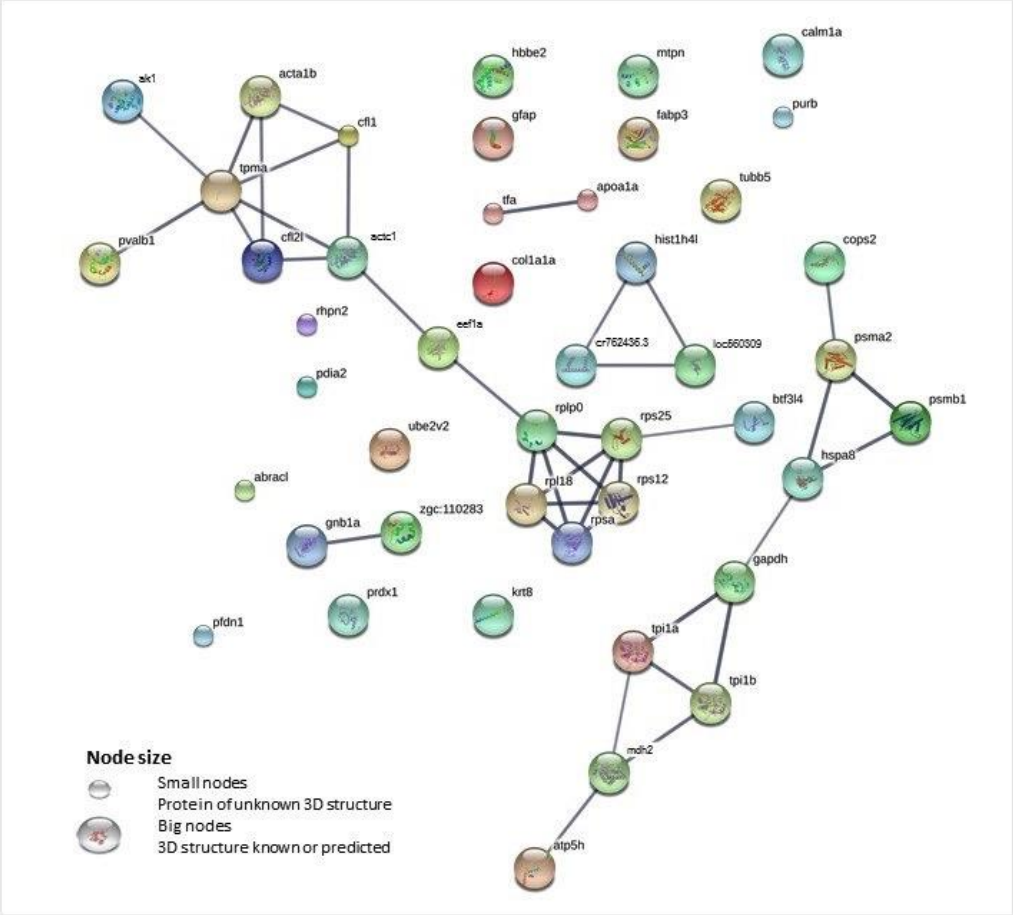


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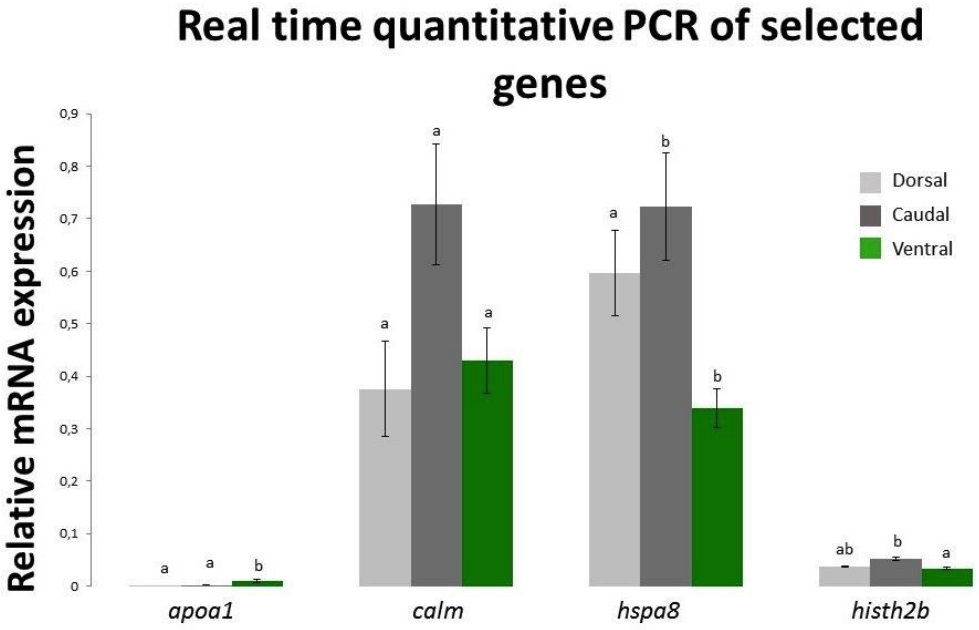
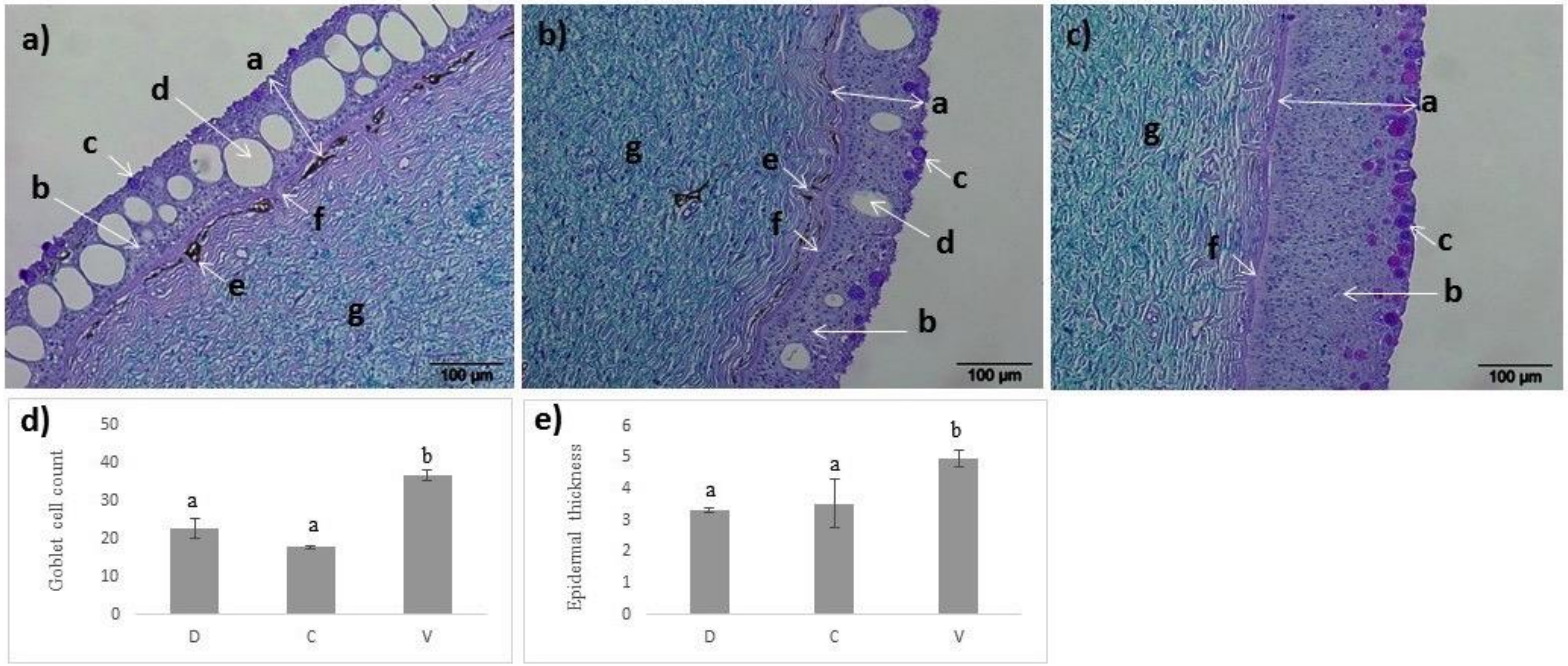
Fig 5



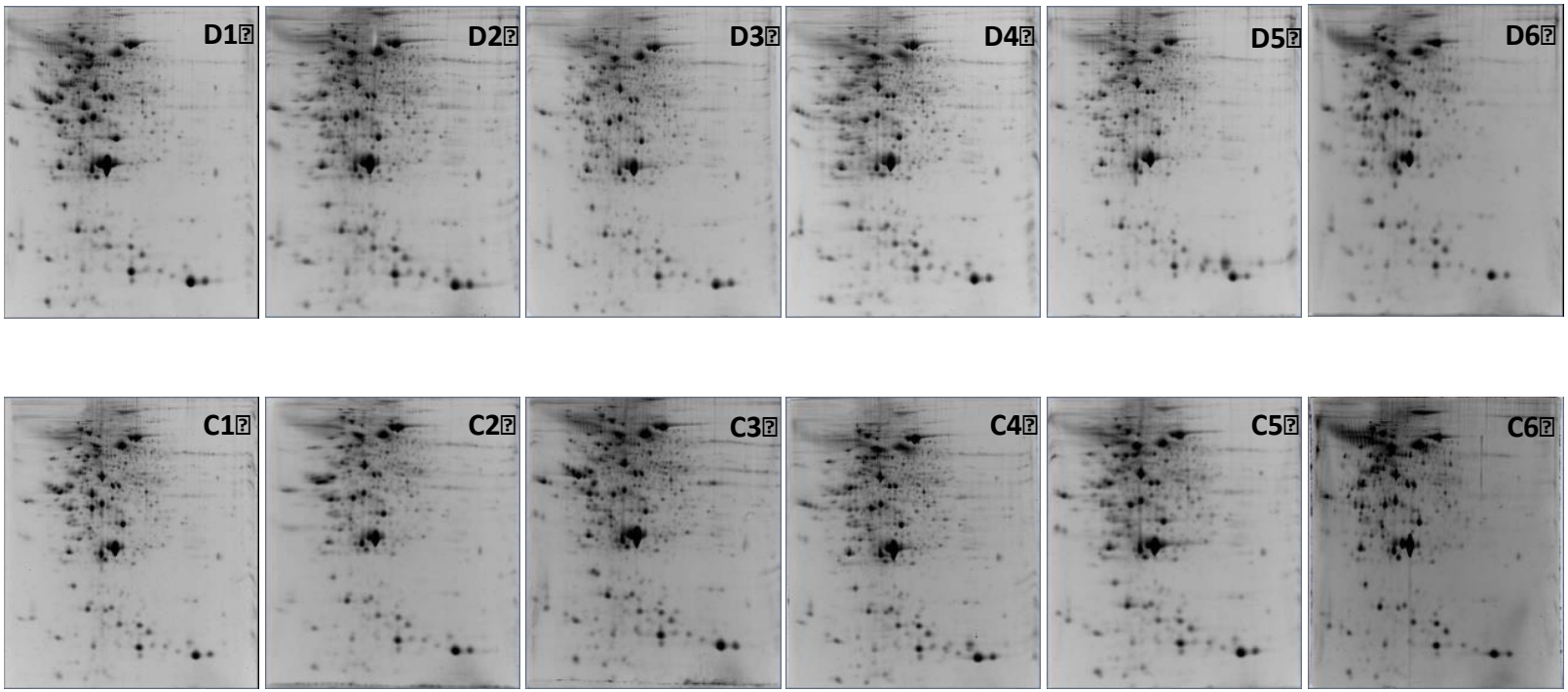
709

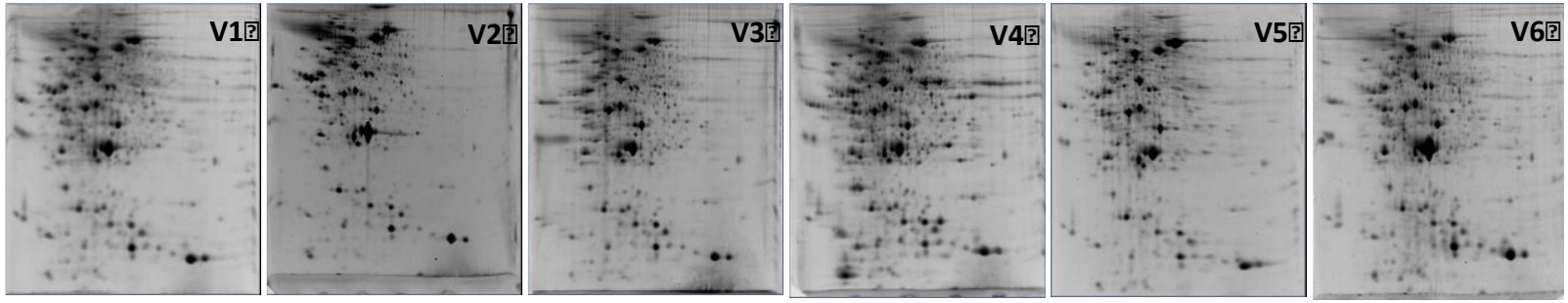
Fig 6


a)
a
d
c
b
f
e
g
100 µm
b)
a
g
e
c
f
d
b
100 µm
c)
a
g
f
c
b
100 µm
d)
Goblet cell count
50
40
30
20
10
0
a
a
b
D
C
V
e)
Epidermal thickness
6
5
4
3
2
1
0
a
a
b
D
C
V

710

Supplementary Figure 1. Images of 2D gels used for analysis in this study.
Figure D1-D6 are gels from the dorsal region, C1-C6 are gels from caudal region and V1-V6 are gels from the ventral region.





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Supplementary Table S1. Abbreviation of protein names used in protein interaction map in Fig. 4 (All abbreviations are assigned by string v.10)

Protein name	Abbreviation
Prefoldin 1	pfdn1
Glial fibrillary acidic protein	gfap
40S ribosomal protein S12	rps12
Fatty acid binding protein	fabp3
Ubiquitin conjugating enzyme E2	ube2v2
Haemoglobin subunit beta	hbbe2
Parvalbumin beta-2	pvalb2
Collagen alpha-1	col1a1a
Keratin cytoskeletal protein	krt8
Calmodulin	calm1a
Histone H2A	loc560309
Histone H2B	cr762436.3
Histone H4	hist1h4l
Myotrophin	mtpn
Apolipoprotein A1	apoa1a
Actin, Actin, heart	acta1b, actc1
Transcriptional activator protein Pur-beta	purb
Heat shock cognate 71 kDa	hspa8
Transferrin	tfa
Serotransferrin	tfa
Triosephosphate isomerase B	tpi1b
Adenylate kinase isoenzyme 1	ak1
Protein disulfide-isomerase	pdia2
Cofilin-1	cfl1
Transcription factor BTF3 homolog 4	btf3l4
40S ribosomal protein S25	rsp25
ATP synthase subunit d	atp5h
Proteasome subunit alpha	psma2
60S acidic ribosomal protein P0	rplp0
Guanine nucleotide-binding protein	gnb1a
costars family protein ABRACL	abracl
Elongation factor 1-alpha	eef1a2
Tubulin beta-1	tubb5
Glyceraldehyde-3-phosphate dehydrogenase 2	gapdh
60S ribosomal protein L18	rpl18
Rhophilin-2	rhpn2
Tropomyosin alpha-1	tpma
40S ribosomal protein SA	rpsa
Malate dehydrogenase	mdh2
Annexin A1	zgc:110283
Triosephosphate isomerase B	tpi1b
COP9 signalosome complex subunit 2	cops2
Natural killer enhancing factor	prdx1
Proteasome subunit beta	psmb9a

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Supplementary Table S2. BestKeeper analysis details of three reference genes *gapdh, ef1-alfa, β-actin*

Gene name	***gapdh***	***ef1-alfa***	***β-actin***
n	18	18	18
SD [± Cq]	0.63	0.52	0.71
CV [% Cq]	2.68	2.49	3.64
r	0.930	0.882	0.865
r^2	0.865	0.778	0.748
p value	0.001	0.001	0.001
Ranking	1	2	3

n; number of samples, SD [± Cq]; standard deviation of Cq values, CV [% Cq]; co-efficient of variation as % of Cq values, r; co-efficient of correlation, r^2; co-efficient of determination.

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Paper III

EFFECT OF CHRONICAL STRESS ON THE SKIN PROTEOME OF LUMPFISH, *CYCLOPTERUS LUMPUS*

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Abstract

Background: Fish skin is the largest and outermost organ and serves as primary line of defense against a wide range of stressors. Crowding is a common abiotic stress factor in aquaculture practice that affects growth and survival of fish.

Results: In this study we have applied gel-based proteomics coupled with liquid chromatography and tandem mass spectrometry (LC-MS/MS) to identify differentially expressed proteins in skin tissues of lumpfish under crowding. Calmodulin, guanine nucleotide binding protein subunit beta 2, glutathione-S-transferase Mu 3, fatty acid binding protein, heat shock cognate 70 kDa protein, keratin, histone H4 and 14-3-3 alpha/beta showed differential expression in lumpfish skin at one or several time points during one month period of crowding stress.

Conclusions: The differentially expressed proteins are related to several metabolic pathways and are involved in stress and immune responses. These proteins might work in a direct or indirect manner in attempt to re-establish the cellular homeostasis affected by crowding. Taken together, the results of this study could be helpful in elucidating the stress response in fish at its molecular level.

Keywords

Skin immunity, stress, 2D gel, lumpfish, crowding, innate immunity

Background

Skin is the largest and outermost organ in fish. It serves several biological functions such as osmoregulation, mechanical protection, primary line of defense against pathogens and external stressors [1]..

Crowding is one of the most common abiotic stressors in aquaculture. It affects animal's physiological status and disease resistance, and alters the immune response of the organism and reduces its capacity to fight against diseases [2, 3]. As a response to any stressful event fish releases cortisol to the blood stream under the influence of hypothalamus-pituitary-interrenal axis. This initiates a series of physiological cascades that influences growth, metabolism, osmoregulation, respiratory and immune functions [4].

Measurement of plasma cortisol level is a common method to assess stress levels in most of the vertebrates including fish [5]. Studies on fish has been performed showing cortisol to be a suitable stress indicator under various stress conditions [6]. However, there are very few studies that focused on these physiological stress responses at their molecular level. Stress response is a very complex process that involves several molecular factors. In brief, the response initiates with a sensor that senses the danger signals from the stressors, and relay it to the effectors, which starts the complex signaling pathway to overcome the stress effect [7]. Study of these proteins/genes could boost the knowledge on understanding the stress response. Proteomics has been used to study the effect of environmental stressors such as the effect of osmotic stress on serum proteome of tilapia (*Oreochromis niloticus*) [8], effect of hypoxia on the serum proteome of juvenile Eurasian perch (*Perca fluviatilis*) [9], effect of microcystin-LR on the liver proteome of medaka (*Oryzias latipes*) [10], changes in skin mucus proteome in

chronically stressed gilthead sea bream (*Sparus aurata*) [11, 12], changes in blood plasma and muscle proteome of Atlantic salmon (*Salmo salar*) under crowding stress [13], and effect of crowding stress on physiological state and flesh quality of a crustacean, chinese shrimp (*Feneropenaeus chinensis*) [14]. These studies suggested the involvement of several genes/proteins involved in stress response in fishes exposed to stressors. Some of the proteins are heat shock proteins, apolipoproteins, lectins, complements factors, several antimicrobial proteins, enzymes and acute phase proteins [11, 12, 15]. However, very little have been explored in terms of change in skin protein expression levels of teleosts under prolonged crowding stress.

Our aim of the paper is to identify differentially expressed skin proteins in lumpfish induced by crowding that has a potential to be used as stress markers in fish welfare and health management.

Materials and methods

Fish rearing

Arctic Cleaner Fish AS, Stamsund, Norway, provided the fishes used in the study. The fish arrived as newly hatched fry and were maintained at 8 ± 0.4°C and oxygen level was 85 ± 3 % O_2 saturation at Mørkvedbukta Research Station, Nord University, Bodø, Norway. The lumpfish were acclimatized for one month before the start of the experiment. The fishes were start fed with Gemma micro feed (50-400 μm, dry pellet) and later fed with Amber Neptun ST pellet (1 μm) depending on the size of lumpfish. Both the feeds used in this study were from Skretting, Norway.

Experimental design and tissue sampling

Approximately 300 lumpsuckers were divided in two tanks (1 m^3) supplied with seawater from 50m depth (34ppt). Fishes were divided into two groups; control and stressed (150 fish per tank). The stressed group experienced daily crowding stress (260 kg/m^3) for a month by lowering water to 0 m and refilling the tank again. It took 6.45 min to drain the water and 14-15 min to refill summing up to approximately 21 min of exposure to the stressor. The stressor was applied randomly every day between 8 am to 4 pm. This was done to ensure that lumpfish did not acclimate to the stressor. The control group did not experience any stress. The fishes were anaesthetized with metomidate solution (5mg/l) and killed with a blow to the head before sampling. To document changes in protein expression (*n*=6) and real time experiment (*n*=5), skin samples (near the operculum above the lateral line) were collected at 7, 14, 21, 28 days of stress. The fishes were anaesthetized by metomidate solution (5 mg/l) and killed by a blow to the head before sampling. The sampled tissues were flash frozen in liquid nitrogen and moved to -80°C immediately after sampling.

Sample preparation and 2DE analysis

Skin tissues were thawed and homogenized under liquid nitrogen using in pestle and mortar. Homogenized samples were dissolved in 1x phosphate buffered saline (Sigma Aldrich, USA) containing (0.1%) protease inhibitor cocktail as per manufacturer's protocol (GE Healthcare, USA). Proteins were extracted and 2DE analysis was performed following the protocol mentioned elsewhere [16]. PDQuest software (BioRad, USA) was used to match the spots in 2D gel images and find differentially expressed proteins. Differentially expressed protein spots were excised and subjected to

LC- MS/MS. The LC- MS/MS analysis was performed at University of Tromsø, Norway.

Protein identification and gene ontology terms

The files obtained from mass spectrometry analysis were blasted in MASCOT for protein identity. The specifications used in MASCOT are maximum 1 missed cleavage allowed, fixed carbamidomethyl (C) modification, variable oxidation (M) modification, 100 ppm of peptide tolerance, 0.1 Da of MS/MS tolerance, monoisotopic, 2+ and 3+ peptide charge and enzyme trypsin. The amino acid sequences were mapped against protein database SwissProt (556196 sequences; 199341870 residues, 06.12.2017) and NCBInr (135744157 sequences; 49805139192 residues, 06.12.2017) restricting the search to Actinopterygii (5307 sequences in SwissProt, 2069668 sequences in NCBI as on 06.12.2017). The gene ontology terms were retrieved from UniProt KB.

Statistical analysis

The data sets were tested for normality using a Kolmogorov-Smirnov test, and for homogeneity of variance using a Levene test. Spot intensities from each protein from stressed group were compared with control group at 7d, 14d, 21d, and 28d using unpaired t-test. Mann Whitney U test was performed wherever data did not show normal distribution.

A one-way ANOVA test was thereafter performed to find the expression trend of proteins in the stressed group and control group. Tukey HSD (Honest Significant Difference) test was used for post hoc analysis ($p<0.05$, $n = 6$). A Kruskal-Wallis ANOVA (non-parametric) and a Mann-Whitney U-test with a Bonferroni-adjusted

significance level were used when requirements for parametric statistics were not met. Significant differences were established at 0.05 levels. Results are given as means ± standard error (SE). All tests were performed in GraphPad Prism Software (GraphPad Software Inc, CA, USA).

Real time quantitative PCR of *hspa8* gene

We performed real time quantitative PCR of the *hspa8* gene to assess the mRNA expression level locally in skin. The RNA was extracted using Omega Bio-tek® EZNA Total RNA extraction kit using the manufacturer's protocol. The RNA integrity was assessed by viewing intact 18S and 28S bands on 1% agarose gel. The quantification was performed in Qubit Fluorometer using Qubit RNA BR assay (Thermo Scientific, USA). The reverse transcription was done using Quantitect Reverse Transcription kit (Qiagen, Germany) using 100 ng of extracted total RNA as input material. The complementary DNA (cDNA) was 5 times diluted and real time PCR was performed on Lightcycler® 96 instrument (Roche, Switzerland) using Sybr green chemistry (Fast start Universal SYBR green, Roche, Switzerland). Fourty five cycles of PCR were run following a preincubation (95 °C for 10 min), two step amplification (95 °C for 10s, 60 °C for 30s), and melting (95 °C for 10s, 65 °C for 60s, 95 °C for 1s). A threefold serially diluted cDNA (prepared from pooled RNA) was used to obtained the standard curve for calculation of PCR efficiency. The formula used for calculation of PCR efficiency was E = 10^(-1/slope) [17]. Beta actin (*β- actin*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were used as reference genes in the study. Primers used in this study were from a previous study on lumpfish (Patel et al., unpublished, Manuscript II in this thesis) (Table 1). All plates were run with no template control,

positive control (pooled cDNA from all samples) and no reverse transcriptase control. The stability of reference genes were estimated using excel based tool Bestkeeper [18]. We used $2^{-\Delta\Delta Ct}$ method for calculation of hspa8 expression [19]. Mann Whitney U test with Bonferroni correction was performed to find the differences between the control and stressed group on day 7, 14, 21 and 28. To find the differences between stressed group among all the four-time points, we used Kruskal Walli's non parametric test. The control groups were also tested using Kruskal Walli's test to find the differences. P value was less than 0.05 and $n = 5$.

Results and discussion

Stress can impair growth and suppress the immune system in fish if it persists for longer periods [20]. Fishes have evolved mechanisms to cope with stress, where a series of proteins and genes are involved in the stress response to combat the unpleasant condition. Proteomic methods can help to identify potential stress markers for a wide range of stressors. 2D gel based proteomics along with homology search to identify the expressed proteins is a suitable technique to study effects of stress in non-model species like lumpfish with very little genomic information available in public databases [21].

The aim of this study was to find the response of the skin proteome of the cold-water marine fish lumpfish under chronic crowding stress. We analyzed the effect of prolonged crowding stress on skin of lumpfish using two-dimensional gel and mass spectrometry based approaches. The finding includes 8 differentially expressed ($p<0.05$) proteins. The proteins are calmodulin, guanine nucleotide binding protein subunit beta 2, glutathione-S-transferase Mu 3, fatty acid binding protein, heat shock cognate 70 kDa protein, keratin, histone H4 and 14-3-3 alpha/beta (Fig 1 and 2). The protein identities

are mentioned in Table 2. Gene ontology terms for these proteins were retrieved from their zebrafish or human orthologues from UniProtKB database. The analysis showed that the differentially expressed proteins are involved in phagocytosis and engulfment (GO:0006911), calcium ion binding (GO:0005509), transport of molecules (GO:0006810), metabolic process (GO:0008152), regulation of protein localization (GO:0032880), keratinization (GO:0031424), chaperoning activity (GO:0061684), nucleosome activity (GO:0006334) and extrinsic apoptotic signaling pathway (GO:0097191).

Differentially expressed proteins in lumpfish skin under crowding

Spot 1 was identified as calmodulin. It is a highly conserved calcium binding protein. Calmodulin is a multifunctional sensor protein that has a role in various biological processes such as inflammation, apoptosis, autophagy, immune and stress response [22-24]. This protein was over expressed in notothenoid fishes of Antarctica exposed to cold stress showing its involvement in stress response [25]. Calmodulin showed a significant increase in expression level in the stressed fish compared to unstressed ones on 14 days and 21 days (Fig 2). Calcium is an essential secondary messenger of several signaling pathways. Calmodulin binds to calcium and provides it to the proteins that are unable to bind to calcium by themselves [26]. Hence, the increase in calmodulin levels in stressed fish suggests that calmodulin dependent signaling pathways might be initiated as a stress response.

Spot 2 was fatty acid binding protein heart-like (Fig 1). This protein was significantly upregulated at 21 days and 28 days in the stressed group compared to the control (Fig 2). An increasing trend of expression in stressed fish was observed from 7 days towards

28 days (Fig 2). This protein is involved in energy metabolism for transportation of lipid. Relative high expression of this protein in the stressed group could be to meet the increased energy requirement due to stress.

Interestingly, inhibition of fatty acid binding protein and resulting peroxisome proliferator-activated receptor α downregulation in zebrafish liver gave increased oxidative stress [27]. This suggest that upregulation of fatty acid binding protein could be important for direct stress mitigation. However, Fatty acid binding protein has also been found to increase inflammation [28] and to be highly expressed in inflammatory diseases [29, 30].

Spot 4 was heat shock cognate 70 kDa protein. This protein is also known as heat shock cognate 71kDa or hsp73 [31]. Heat shock proteins/genes expressed in fishes exposed to stressors such as temperature, salinity, crowding, pathogenic infections, chemicals and toxins [32-34]. Hsc70 is a multifunctional protein involved in inflammation, infection, autophagy, chaperoning activity, protein folding and protein degradation [35]. A short term crowding stress (100g/l) of 24 hours in Wuchang bream (*Megalobrama amblycephala*) showed downregulation of hsc70 mRNA at first and then it was upregulated in the liver to maintain the homeostasis [33]. In the present study the hsc70 protein in the stressed group showed a consistent downregulation in expression compared to the control groups at all time points. Downregulation of hsc70 in all time points in this study could be due to the long period of exposure to crowding where the fish could not regain the homeostasis. This is also confirmed by physiological data that showed the stressed group after 14 days showed signs of allostatic overload type 2 (chronical stress) due to oversensitivity to ACTH, and a reduced negative feedback

system with increased baseline levels of cortisol as result (Hanssen et al, Unpublished, Manuscript IV in this thesis) [36].

Spot 5 was glutathione-S-transferase Mu 3 protein. It is an enzyme from the large glutathione-S-transferase (GST) family with detoxification of electrophiles as its primary function. In many organisms expression of GSTs has been observed due to oxidative stress [37]. Glutathione-S-transferase Mu in mice can modulate the stress activated signaling by suppressing the expression of apoptosis signal regulating kinase that serves as defense against oxidative stress [37]. This protein was upregulated at 21 days and a significant difference was observed between 21 days and 28 days in stressed group.

Spot 7 was identified as histone H4 protein. The primary function of histone proteins is the major role in DNA condensing in the nucleosome complex but they have also antimicrobial functions [31]. The nucleosome complex contains the major histone proteins (H2A, H2B, H3 and H4) and several non-histone proteins are associated with it for DNA packaging. Under stressful conditions the DNA packaging might get affected, e.g. as part of regulation of gene expression and so the proteins involved in it might get regulated differently regulated and/or expressed compared to non- stressed individuals [7, 38]. In channel catfish skin the expression levels of histone like proteins (HLP) were suppressed in stressed (overcrowding and high ammonia levels) fish compared to unstressed fish. The unstressed fish showed a consistent higher level of HLP for week 1 till week 4 of the experiment than the stressed fish [31, 39]. In our study, the lumpfish skin showed high level of histone H4 protein in both stressed and unstressed fish till 21d. But, on 28d the stressed fish showed a significant level of drop in the expression of histone H4 compared to the unstressed fish. This could have influence the gene

242 expression, but also the role of histones as antimicrobial proteins. As long term
243 exposure to stress impairs the immune system of organism [40], the relatively low
244 expression on histone H4 on 28d could indicate a suppression of the immune system in
245 the stressed group.
246 Spot 8 was keratin type-II cytoskeletal 8 like protein. The expression of this protein was
247 upregulated by 21d and then downregulated by 28d in lumpfish skin due to crowding.
248 This protein was found to be downregulated in skin mucus of stressed (random light
249 flashes, shaking of tanks, random sounds, moving objects into water, water reverse flow
250 in random order for 30 min three times per day for 21 days) gilthead sea bream [11].
251 Keratins are involved in translational activity, phagocytosis, cell size determination and
252 stress responses. Keratin performs these functions by its highly dynamic scaffolding
253 activity [41]. A study on human suggested that keratin could, in addition to its structural
254 role, protect the tissue from injury by removing the stress activated kinases by acting as
255 a phosphate sponge [42]. Keratin has also been shown to have antimicrobial activity
256 [43]. In the current study, we speculate that, the upregulation of keratin by 21d day
257 could be to remove the stress induced kinases but as the crowding still continued after
258 21 days, the fish might not be able to recover to long exposure to stress and thus the
259 keratin expression reduced on 28d compared to 21d (Fig 2).
260 In conclusion, we identified proteins that were expressed differently in response to
261 crowding stress. Gene ontology analysis showed the involvement of the expressed
262 proteins in metabolic pathways, binding, protein regulation, phagocytosis and
263 engulfment. Among all the expressed proteins hsc70 could be a potential stress marker
264 in skin for crowding stress as it showed a differential trend at all time points compared

to the non-stressed group. Further research on pathway analysis of the identified protein would add to the existing knowledge of stress responses in fishes.

Expression of *hspa8* gene in lumpfish skin subjected to prolonged stress

The expression level of *hspa8* gene was relative to the geometric mean of two reference genes *beta-actin* and *gapdh.* Both the reference genes showed stable expression with a p value of 0.001 and correlation coefficient value (*r*) 0.0969 (*beta-actin*) and 0.974 (*gapdh*) (Table 3). Therefore we used the geometric mean of both reference genes for the analysis of the *hspa8* gene expression. The *hspa8* gene was significantly upregulated in stressed group on day 7, but did not show significant difference on day 14, 21 and 28. The control groups did not show any significant difference. The stressed group on day 7 was significantly different compared to the stressed group of day 28. The results indicate that there is an increase in level of *hspa8* gene expression on day 7, 14, and 21 compared to control group but on day 28 the level of expression reduced. This could be due to the chronic stress effect, as the *hspa8* gene is known to show differential expression due to stress. The expression level of *hspa8* gene did not correlate with the protein expression levels of heat shock cognate 70kDa. However, the protein and mRNA expression level do not always correlate, as seen in e.g. one study performed on yeast, *Saccharomyces cerevisiae* [44].

Declarations

Ethics approval and consent to participate

The experiment described has been approved by the local responsible laboratory animal science specialist under the surveillance of the Norwegian Animal Research Authority

(NARA), Norway and was approved by the Norwegian Food Safety Authority (in the FOTS system) registered by the Authority with approval number 7293, 2015

Consent for publication

Not applicable

Availability of data and material

The 2D gel images are supplied as additional file. Please contact author for any further material or data required.

Competing interests

The authors declare no competing interests.

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Authors' contributions

MHI conceived the study and designed the experimental set up for wet lab. MFB designed the proteomics analysis of fishes under crowding stress. DMP sampled the tissues, performed all experiments and wrote the first draft. All authors reviewed the manuscript.

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References

1. Esteban MA. An Overview of the immunological defenses in fish skin. ISRN Immunol. 2012; 2012:1-29.

2. Ortuño J, Esteban MA, Meseguer J. Effects of short-term crowding stress on the gilthead seabream (Sparus aurata L.) innate immune response. Fish Shellfish Immunol. 2001; 11(2):187-97.

3. Iversen MH, Eliassen RA. The effect of allostatic load on hypothalamic–pituitary–interrenal (HPI) axis before and after secondary vaccination in Atlantic salmon postsmolts (Salmo salar L.). Fish Physiol Biochem. 2014; 40(2):527-38.

4. Iwama GK. Stress in Fish. Ann NY Acad Sci. 1998; 851(1):304-10.

5. Ellis T, Yildiz HY, López-Olmeda J, Spedicato MT, Tort L, Øverli Ø, Martins CIM. Cortisol and finfish welfare. Fish Physiol Biochem. 2012; 38(1):163-88.

6. Endocrine stress response in Oreochromis sp. from exposure to waterborne cadmium: the plasma cortisol analysis. Toxico Environ Chem. 2017; 99(2):285-93.

7. Smith KT, Workman JL. Chromatin Proteins: Key Responders to Stress. PLoS Biology. 2012; 10(7):e1001371.

8. Kumar VB, Jiang I-F, Yang H-H, Weng C-F. Effects of serum on phagocytic activity and proteomic analysis of tilapia (Oreochromis mossambicus) serum after acute osmotic stress. Fish Shellfish Immunol. 2009; 26(5):760-67.

9. Douxfils J, Deprez M, Mandiki SNM, Milla S, Henrotte E, Mathieu C *et al*. Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication – Clues to physiological acclimation and humoral immune modulations. Fish Shellfish Immunol. 2012; 33(5):1112-22.

10. Karim M, Puiseux-Dao S, Edery M. Toxins and stress in fish: Proteomic analyses and response network. Toxicon. 2011; 57(7):959-69.

11. Pérez-Sánchez J, Terova G, Simó-Mirabet P, Rimoldi S, Folkedal O, Calduch-Giner JA, Olsen RE, Sitjà-Bobadilla A. Skin mucus of gilthead sea bream (*Sparus aurata* L.). Protein mapping and regulation in chronically stressed fish. Front Physiol. 2017; 8:34.

12. Cordero H, Morcillo P, Cuesta A, Brinchmann MF, Esteban MA. Differential proteome profile of skin mucus of gilthead seabream (*Sparus aurata*) after probiotic intake and/or overcrowding stress. J Proteomics. 2016; 132:41-50.

13. Veiseth-Kent E, Grove H, Færgestad EM, Fjæra SO. Changes in muscle and blood plasma proteomes of Atlantic salmon (Salmo salar) induced by crowding. Aquaculture. 2010; 309(1–4):272-79.

14. Zhang S, Fu L, Wang Y, Lin J. Alterations of protein expression in response to crowding in the Chinese shrimp (Fenneropenaeus chinensis). Aquaculture. 2014; 428–429:135-40.

15. Ni M, Wen H, Li J, Chi M, Ren Y, Song Z, Ding H. Two HSPs gene from juvenile Amur sturgeon (Acipenser schrenckii): cloning, characterization and expression pattern to crowding and hypoxia stress. Fish Physiol Biochem. 2014; 40(6):1801-16.

16. Patel DM, Brinchmann MF. Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). Biochem Biophys Rep. 2017; 9:217-25.

17. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 2001; 29(9):e45-e45.

18. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004; 26(6):509-15.

19. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods. 2001; 25(4):402-08.

20. Barton BA. Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids1. Integrative and Comparative Biology. 2002; 42(3):517-25.

21. Junqueira M, Spirin V, Balbuena TS, Thomas H, Adzhubei I, Sunyaev S, Shevchenko A. Protein Identification Pipeline for the Homology Driven Proteomics. J Proteomics. 2008; 71(3):346-56.

22. Antimicrobial activity in the skin of the channel catfish Ictalurus punctatus-characterization of broad-spectrum histone-like antimicrobial proteins.

23. Berchtold MW, Villalobo A. The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2014; 1843(2):398-435.

24. Li S, Jia Z, Li X, Geng X, Sun J. Calmodulin is a stress and immune response gene in Chinese mitten crab *Eriocheir sinensis*. Fish Shellfish Immunol. 2014; 40(1):120-28.

25. Yang N, Peng C, Cheng D, Huang Q, Xu G, Gao F, Chen L. The over-expression of calmodulin from Antarctic notothenioid fish increases cold tolerance in tobacco. Gene. 2013; 521(1):32-37.

26. Chin D, Means AR. Calmodulin: a prototypical calcium sensor. Trends in Cell Biology. 2000; 10(8):322-28.

27. Zhang Y, Liu K, Hassan HM, Guo H, Ding P, Han L *et al*. Liver fatty acid binding protein deficiency provokes oxidative stress, inflammation, and apoptosis-mediated hepatotoxicity induced by pyrazinamide in zebrafish larvae. Antimicrob Agents Ch. 2016; 60(12):7347-56.

28. Hui X, Li H, Zhou Z, Lam KSL, Xiao Y, Wu D *et al*. Adipocyte Fatty Acid-binding Protein Modulates Inflammatory Responses in Macrophages through a Positive Feedback Loop Involving c-Jun NH(2)-terminal Kinases and Activator Protein-1. The Journal of Biological Chemistry. 2010; 285(14):10273-80.

29. Yeung DCY, Xu A, Tso AWK, Chow WS, Wat NMS, Fong CHY, Tam S, Sham PC, Lam KSL. Circulating Levels of Adipocyte and Epidermal Fatty Acid–Binding Proteins in Relation to Nephropathy Staging and Macrovascular Complications in Type 2 Diabetic Patients. Diabetes Care. 2009; 32(1):132-34.

30. Milner K-L, van der Poorten D, Xu A, Bugianesi E, Kench JG, Lam KSL, Chisholm DJ, George J. Adipocyte fatty acid binding protein levels relate to inflammation and fibrosis in nonalcoholic fatty liver disease. Hepatology. 2009; 49(6):1926-34.

31. Robinette D, Wada S, Arroll T, Levy MG, Miller WL, Noga EJ. Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. Cell Mol Life Sci. 1998; 54(5):467-75.

32. Ellison MA, Ferrier MD, Carney SL. Salinity stress results in differential Hsp70 expression in the Exaiptasia pallida and Symbiodinium symbiosis. Marine Environmental Research. 2017; 132(Supplement C):63-67.

33. Ming J, Xie J, Xu P, Liu W, Ge X, Liu B *et al*. Molecular cloning and expression of two HSP70 genes in the Wuchang bream (Megalobrama amblycephala Yih). Fish Shellfish Immunol. 2010; 28(3):407-18.

34. Boone AN, Vijayan MM. Constitutive heat shock protein 70 (HSC70) expression in rainbow trout hepatocytes: effect of heat shock and heavy metal exposure. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 2002; 132(2):223-33.

35. Stricher F, Macri C, Ruff M, Muller S. HSPA8/HSC70 chaperone protein: structure, function and chemical targeting. Autophagy. 2013; 9(12):1937-54.

36. Hanssen AB, Patel DM, Brinchmann MF, Iversen MH. The effect of long-term stress on basal levels of plasma cortisol and hypothalamic–pituitary–Interrenal (HPI) Axis in lumpsucker (Cyclopterus lumpus). Unpublished.

37. Cho S-G, Lee YH, Park H-S, Ryoo K, Kang KW, Park J *et al*. Glutathione S-Transferase Mu Modulates the Stress-activated Signals by Suppressing Apoptosis Signal-regulating Kinase 1. J Biol Chem. 2001; 276(16):12749-55.

38. Rando OJ. Combinatorial complexity in chromatin structure and function: revisiting the histone code. Current opinion in genetics & development. 2012; 22(2):148-55.

39. David WR, Edward JN. Histone-like protein: a novel method for measuring stress in fish. Dis Aquat Organ. 2001; 44(2):97-107.

40. Tort L. Stress and immune modulation in fish. Dev Comp Immunol. 2011; 35(12):1366-75.

41. Magin TM, Vijayaraj P, Leube RE. Structural and regulatory functions of keratins. Experimental Cell Research. 2007; 313(10):2021-32.

42. Ku N-O, Omary MB. A disease- and phosphorylation-related nonmechanical function for keratin 8. The Journal of Cell Biology. 2006; 174(1):115-25.

43. Molle V, Campagna S, Bessin Y, Ebran N, Saint N, Molle G. First evidence of the pore-forming properties of a keratin from skin mucus of rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri). Biochem J. 2008; 411(1):33.

44. MacKay VL, Li X, Flory MR, Turcott E, Law GL, Serikawa KA *et al*. Gene Expression Analyzed by High-resolution State Array Analysis and Quantitative Proteomics: Response of Yeast to Mating Pheromone. Molecular & Cellular Proteomics. 2004; 3(5):478-89.

Table 2. Differentially expressed proteins from skin of lumpsucker under crowding stress

Spot ID	Protein name	Organism (Accession number)	ST[a]/ SC[b] (%)	Total score/score of Up[c]	MW/pI from MASCOT	Peptide sequences
1	Calmodulin	*Ctenopharyngodon idella* (Q6IT78)	20/11	41/41	16827/4.09	KEAFSLFDKDGDGTITTKE
2	Fatty acid binding protein	*Maylandia zebra* (XP_004549259)	49/24	179/119	15269/6.34	KAIGVGFATRQ KCIMGDVIAVRT KLNEPFDETTADDRKT
3	14-3-3 protein beta/alpha-1 like	*Seriola dumerili* (XP_022594810)	57/18	442/152	27711/4.62	RYLSEVASGDTKK RYLSEVASGDTKKD KSVTEQGAELSNEERN KDTVENSQQAYQQAFDISKG KKDTVENSQQAYQQAFDISKG
4	Heat shock cognate 70 kDa	*Onchorhynchus mykiss* (P08108)	23/7	236/236	71581/5.24	KVEIIANDQGNRT RIINEPTAAAIAYGLDKK KSINPDEAVAYGAAVQAAILSGDKS
5	Glutathione-S-transferase Mu 3	*Anoplopoma fimbria* (ACQ58211)	57/7	139/139	25955/6.84	KLAYWDIRG RLLLEYTGSKY
6	Guanine nucleotide binding protein subunit beta 2 like	*Danio rerio* (O42248)	29/2	57/57	35557/7.60	RVWQVTIGTR
7	Histone H4	*Larimichthys crocea* (KKF20647)	49/15	132/132	15136/11.48	RISGLIYEETRG RDNIQGITKPAIRR
8	Keratin, type II cytoskeletal 8-like	*Scleropagus formosus* (KPP69326)	56/3	141/141	55997/5.22	RTLLEGEESRL KLALDIEIATYRX

[a]Significant threshold, [b]Sequence coverage, [c]Unique peptide, Unique peptide sequences are in red.

445

446

Table 2. Primers used in this study

Gene name	Primer sequence	Amplicon size	Primer efficiency (%)
hspa8	F: TCTCATTGGACGTCGGTTT R: TGGTCTCGCCCTTGTACTC	119	109.54
gapdh	F: GGGGCAAGCTCATCGTCG R: CCTGGATGTGAGAGGAGGCC	149	108.68
beta-actin	F: GACTACCTCATGAAGATCCTGA R: GGTGATGACCTGTCCGTC	188	109.18

Table 3. Bestkeeper analysis for stability of reference genes

	beta-actin	***gapdh***
coeff. of corr. [r]	0.969	0.974
coeff. of det. [r^2]	0.939	0.949
intercept [CP]	0.77	-0.7902
slope [CP]	0.9283	1.074
SE [CP]	±0.247	±0.26
p-value	0.001	0.001
Power [x-fold]	1.903032236	2.105262309

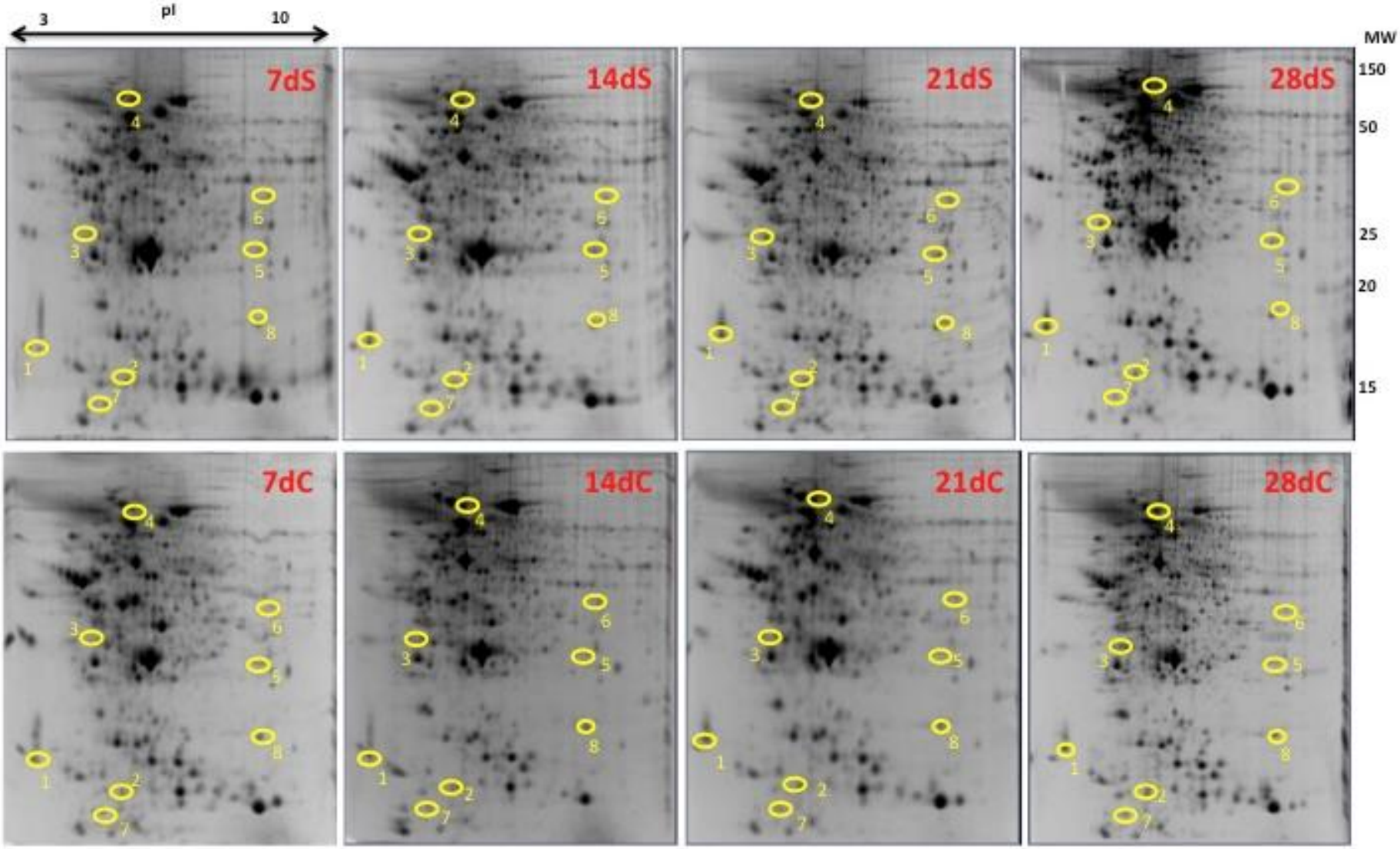
Figure legends

Fig 1. Gel images from control and stressed groups showing differentially expressed proteins. Polyacrylamide gel (12.5%) images from 7, 14, 21, 28 day showing identified proteins from stressed and control fishes. C stands for control and S stands for stressed group. Each gel represents protein from one fish. Proteins differentially expressed are encircled in yellow and denoted by numbers. 1- calmodulin, 2- fatty acid binding protein, 3- 14-3-3 alpha/beta, 4- heat shock cognate 70, 5- glutathione-S- transferase, 6- guanine nucleotide binding protein, 7- histone H4 and 8- keratin. Molecular weight (MW) measured in kDa.

Fig 2. Protein expression level of identified proteins in control and stressed group. Figure shows the protein expression values as spot intensity given by PDQuest (Y-axis) during the course of the experiment measured on day 7, 14, 21, and 28 (X-axis). Error bar shows error of mean ($p < 0.05$, $n = 6$). Letters signifies the difference in expression between

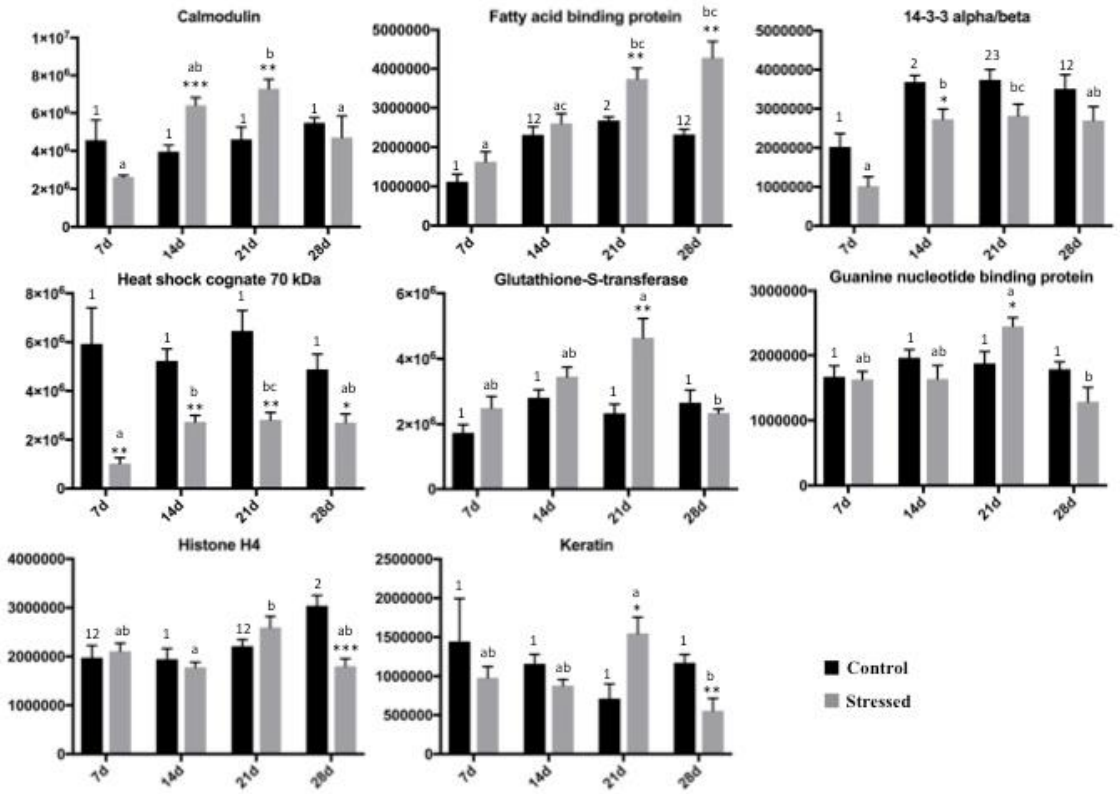
463 the stressed group and number signifies the difference in expression between the control
464 group along the course of experiment at different time points (One way anova and Tukey
465 HSD post hoc analysis; Kruskal Walli's test and Dunn's test for nonparametric data). Similar
466 number/ letters shows no difference and different number/letters indicates a difference.
467 Asterisk shows the comparison between the control and stressed fish at each time point
468 (unpaired t-test for data the showed normal distribution and Mann Whitney U test for data
469 that did not show normal distribution).
470
471 **Figure 3. Relative mRNA expression of *hspa8* gene.** The expression is relative to the
472 geometric mean of two reference genes *beta-actin* and *gapdh* (glyceraldehyde 3-phosphate
473 dehydrogenase). X-axis shows the time point in days 7, 14, 21, and 28. Asteriks denote a
474 significant difference between the control and stressed group on day 7. The letters refers to
475 the changes in expression between the stressed group on day 7 til 28. The number shows
476 difference in expression between the control groups from day 7 til 28. Similar letters and
477 number means no change and vice versa. Error bar shows error of the mean, $n = 5$, $p < 0.05$.
478 Details about statistical analysis is mentioned in section
479

Fig 1



480

481 **Fig 2.**



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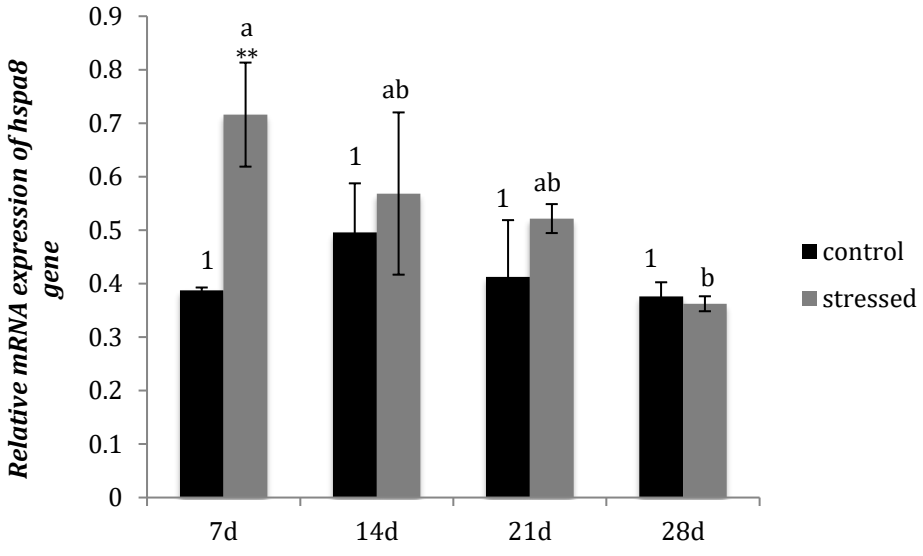
483

484 **Fig 3.**



485

Paper IV

The effect of chronic stress on primary, secondary and tertiary stress responses, and its impact on animal welfare in lumpfish (*Cyclopterus lumpus*).

Running title: Chronic stress in lumpfish

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Abstract

The purpose of this study was to study the response of the hypothalamic–pituitary–Interrenal (HPI) axis during exposure to long-term crowding stressor in lumpfish (*Cyclopterus lumpus*). The experiment consisted of two groups. Group 1: Daily crowding stress for one month and group 2: Control (no stress). Blood samples were obtained prior to the start of the experiment (pre-stress), and after 7, 14, 21, 28 days of stress (n=6), and at the same sampling time adrenocorticotropic hormone (ACTH) stimulation and dexamethasone (DEX) suppression test were conducted. The results indicated that stress group showed signs of allostatic overload type 2 (chronic stress) due to oversensitivity to ACTH, and a reduced negative feedback system with increased baseline levels of cortisol as a result. These chronic changes in the endocrine system promoted changes in secondary and the tertiary stress responses as reduced osmoregulatory capacity and stunted growth, after 14 days of stress and onward. These results show that the robust lumpfish can suffer from chronic stress with possible dire consequences for the animal welfare. Thus, care should be taken to reduce the overall stress during commercial production of lumpfish aquaculture industry.

Keywords: Lumpfish, chronic stress, allostatic load, welfare

1. Introduction

Norway is well suited for fish farming, with its long coastline with fjords and islands, which provides calm and protected environment. In aquaculture, the Atlantic salmon (*Salmo salar*) represents 90% of the production (Burridge Weis Cabello Pizarro & Bostick, 2010). Some argue that salmon is the most sustainable farmed animal (Mozaffarian & Rimm, 2006), a "super chicken", with a feed ratio of 100 to 65 (dry feed to meat), (Torrissen et al., 2011), whereas others point out that there are also many problems in the production process. Escapees and sea lice are the biggest environmental concern both from a biological, environmental and from the consumer's point of view. The sea lice are marine parasitic copepods that have been a large problem for both wild and farmed fish from the early start of commercial aquaculture (Brandal & Egidius, 1979). Salmon aquaculture has struggled with sea lice for a long time, and the problem has been increasing in the later years (Taranger et al., 2014). The sea lice problem has manifested itself in increased use of chemotherapeutants, with subsequently increased resistance against the therapeutics. Due to this, re-infections of sea lice are a common problem, and effective strategies to control the sea lice infestation are hard to come by (McVicar, 2004; Taranger et al., 2014).

As problems with lice and their resistance to chemical agents have increased, the interest for cleaner fish has increased accordingly (Treasurer, 2002). Already almost 50% of all Norwegian salmon localities use cleaner fish as part of their sea lice control strategy, and the dominant species is lumpfish (*Cyclopterus lumpus*). In 2016, almost 16 million lumpfish were put out to sea and all originated from farmed facilities (DOF, 2017a). Lumpfish is a marine cottoid teleost fish from the family Cyclopteridae (lumpfish) (Davenport 1985). Recent studies have shown that lumpfish is an effective cleaner fish (Norethberg Johannesen & Arge, 2015), grazing on pre-adult and adult stages of sea lice (Imsland et al., 2014). Field studies seem to support these investigations, and suggest that not only the most common salmon lice, *L. salmonis* but also *C. elegans* is a part of the lumpfish diet (Imsland et al., 2014). It has a natural northern distribution, with broad tolerance of temperature and supposedly sturdiness to stress and suboptimal water conditions, which makes it perfect species for use with Atlantic salmon in northern part of Norway (Imsland et al., 2014). However, early reports from the aquaculture industry

indicate that lumpfish suffers from higher mortality rate during the early production phase and seems to tolerate long-term stress poorly (Pers. Communication, Dag Hansen, Arctic Cleanerfish AS, Stamsund, Norway).

Stress is defined as a condition in which the "dynamic equilibrium" of an organism, called homeostasis, is threatened or disturbed as a result of the actions of internal or external stimuli, commonly defined as stressors (Selye, 1950; Selye, 1973; Varsamos Flik Pepin Bonga & Breuil, 2006; Wendelaar Bonga, 2011; Wendelaar Bonga, 1997). Recently, the concept of allostasis has been introduced to complement the concept of stress, and more precisely try to describe the role primary mediators (e.g. glucocorticosteroids) have in response to a stressor (Goymann & Wingfield, 2004; McEwen, 1998; McEwen, 2005; McEwen & Wingfield, 2003; Wingfield, 2005). In the study of McEwen and Wingfield (2003), they considered allostasis to be "*the ability to achieve stability through change.*" This is a process that supports homeostasis, i.e., those physiological parameters essential for life defined above, as environments and/or life history stages change. An allostatic state refers to altered and sustained activity levels of the primary mediators (e.g. glucocorticosteroids) as a response to a stressor (McEwen & Wingfield, 2003). The cumulative result of an allostatic state is allostatic load. Within limits an organism can cope with, adapt to, or tolerate stressors to keep homeostasis. However, when the system is not dealing well with the disturbing factor(s) the increased allostatic load results in allostatic overload, which either could be adaptive (overload type 1) or malicious for the animal (overload type 2) (Juster McEwen & Lupien, 2010; McEwen, 2005; McEwen & Wingfield, 2003; Wingfield, 2005).

However, as far as we know no scientific study has been published on the allostatic load on hypothalamic–pituitary–Interrenal (HPI) axis in lumpfish subjected to a long-term crowding (chronic) stressor. Thus, the focus in this study is on the primary stress response in context of the corticotropic axis and its influence on secondary and tertiary stress responses in lumpfish subjected to chronic stress.

2. Materials and Methods

2.1 Fish and rearing conditions

Lumpfish roe and milt was collected by Arctic Cleanerfish AS (Stamsund, Norway) from a wild, locally caught broodfish, and delivered to Nord University research station (Bodø, Norway) as newly hatched fry before the start of feeding in the end of May 2014. Start feeding was based on Gemma Micro feed (Skretting AS, Norway) 50-400 µm dry feed pellets. After initial feeding the fish were fed Amber Neptun ST (Skretting AS, Norway), the pellet size was increased from 1 mm to 4 mm depending on the size of lumpfish. The fish was kept under 24L:0D cycle.

2.2 Stress experiment and sampling procedures

The experimental tanks were two quadratic 1 m^3 gray fiber-glass, of 500 L rearing volume units. Automatic feeders (Arvo-Tec T Drum 2000 feeder, Arvo-Tec Oy, Finland) were installed on each tank, and a computer program estimated the daily feeding rates to feed in excess.

Filtered seawater (34 ppt) was supplied from 250m depth, and the water was treated with different filters and UV. Water temperature during the experiment was 8 ± 0.4°C and the oxygen level was kept at 85 ± 3 % O_2 saturation. The lumpfish were acclimated for a period of one month prior the start of experiment.

The experiment was conducted on 8 months old fish January to February 2015. Approximately 300 lumpfish were divided in two tanks. The experiment consisted of two groups (150 fish per tank). Group 1: Daily stress for 1 month and Group 2: Control (no stress). Daily routines of flushing excess feed, faeces, and monitoring fish behaviour were enabled in both tanks. Group 1 was exposed to a crowding stressor every day (between 08:00-14:00 every day) for a month, and this was done by lowering of the water level so that dorsal fins were exposed in air by opening of drains. Crowding gave an average density of 265 kg m^{-3}. The average draining time was 6.45 minutes, and normal water level was restored after 14-15 minutes. The total duration of the stressor was approximately 21 minutes.

To document changes in resting levels of plasma cortisol, lactate, glucose, osmolality, chloride and magnesium during the experiment, blood samples were obtained prior to start of the experiment (pre-stress), and after 7, 14, 21, 28 days of stress (n=6). Prior to blood sampling the fish was measured for weight (g) and length (cm). The blood sample was taken monday morning every week at 8 am to ensure that the fish had at least 18 hours rest after the last applied stressor. The fish was anesthetised in 5 mg L^{-1}metomidate in seawater . This concentration has shown to be sufficient in inducing rapid anaesthesia and preventing an increase in blood plasma cortisol (Iversen Finstad McKinley & Eliassen, 2003; Olsen Einarsdottir & Nilssen, 1995). After being killed with a blow to the head, blood from six fish (per group) at each sampling time was drawn from the caudal vein complex using size 0.50-x16-mm heparinised syringes. The blood was centrifuged at 5000 rpm for 5 min at room temperature, plasma was removed and stored in cryo tubes at -36 °C until analyses were performed.

2.3 Measurement of cortisol, osmolality, chloride, magnesium, lactate and glucose.

A radioimmunoassay (RIA) technique was used to measure plasma cortisol concentrations as described by Iversen et al. (1998). Plasma was also analyzed for osmolality and chloride levels using a Wescor 5500 osmo-meter (Wescor®) and a Sherwood Chloride Analyzer 926 (Sherwood Scientific Inc. USA), respectively. Magnesium (Mg^{2+}) was analysed by a fluidtest® Mg-XB (Biocon®, Germany) adapted for plate count reader. Samples below the detection limit were given a value corresponding to the tests sensitivity, which was 0.4 mmol L^{-1} (mM). Lactate and glucose were tested from the whole blood immediately after taking the sample using Lactate Pro™ (Arkray KDK, Kyoto, Japan) and Freestyle Freedom Lite (Abbott Diabetes Care Ltd., Oxon, UK), respectively. Levels of lactate and glucose below detection limit were assigned a value corresponding to the sensor's minimal sensitivity, which was respectively 0.8 mM (lactate) and 1.1 mM (glucose). Use of transportable instruments, for measuring of glucose and lactate, was validated in previous research, gave equivalent results to established laboratory techniques (Wells & Pankhurst, 1999).

2.4 Adrenocorticotropic hormone stimulation and dexamethasone suppression test

Stimulation (adrenocorticotropic hormone) and dexamethasone suppression test was conducted in accordance to Pottinger and Carrick (2001), with some minor modifications. Briefly, at pre-stress, 7, 14, 21 and 28 days after start of the experiment 12 fish per group (a total of 24 fish), were netted from their respective tanks, then anaesthetised (as described in section 2.2), and injected intraperitoneally with 1 mg kg^{-1} dexamethasone (Sigma-Aldrich) in ethanol: phosphate-buffered saline (PBS; 1:3; 1µg μL^{-1}). Finally, they were transferred to two holding tanks (0.5 m^3). After 24 h the fish were netted, anaesthetised, and 6 fish from each group was either given an intraperitoneal injection of 0.5 mL kg^{-1} adrenocorticotropic hormone (ACTH fragment 1-24; Sigma-Aldrich in PBS at 45 µg m L^{-1}) or 0.5 mL kg^{-1} PBS, to measure the function of the negative feedback system. The ACTH and PBS groups were kept separate by an artificial wall inserted in two different holding tanks. Two hours after the ACTH/PBS administration, the fish were netted, anaesthetised and blood sampled. The blood was centrifuged at 5000 rpm for 5 min at room temperature, and plasma was removed and stored in cryo tubes at -36 °C until plasma cortisol analyses were performed.

2.5 Calculation of specific growth rate

Specific growth rate (SGR) was calculated utilising the following equation:

$$\frac{(\ln(final\ weight\ (g)) - \ln(start\ weight\ (g))}{\#\ days} \times 100$$

An average overall SGR was calculated for the experimental period (28 days) for all experimental groups.

2.6 Statistics

SPSS for Windows (ver. 18.00) was used for statistical analyses. Kolmogorov–Smirnov test for normality and Levene's test for homogeneity was performed on all the data. A one-way ANOVA test was thereafter performed at each sampling time in regard to various physiological parameters to test for differences between the test groups and within the groups (Sokal & Rohlf, 1987). If the F-values were significant, a Bonferroni post hoc test

was used to determine which groups differed. A Kruskal-Wallis ANOVA (non-parametric) and a Mann-Whitney U-test with a Bonferroni-adjusted significance level were used when requirements for parametric statistics were not met. Significant differences were established at level 0.05.

3. Results

3.1 Primary stress response

Figure 1 show the changes in resting levels of plasma cortisol during daily stress and no stress (control). The average resting levels of plasma cortisol increased during the experiment in the daily stress group, and become significantly elevated both from pre-stress levels and from control group at the same sampling time at 21 (25.3 ± 10.1 nM) and 28 (22.5 ± 14.1 nM) days after start of the experiment.

Insert Figure 1

3.2 Secondary stress responses

The initial mean glucose levels in the pre-stressed group ranged between 0.7 to 2.1 mM with a average of 1.60 (± 0.59 mM) for both groups. The highest glucose levels were detected in the control group at day 14 at 2.97 mM (± 1.78). There were no significant differences between the sampling groups, neither at the same sampling time or compared to pre-stress levels.

The average concentration of lactate in blood of fish from the control group was 0.77 mM (± 0.08) before the start of the experiment. However, plasma lactate was not measurable (not above detection limit) by the Lactate Pro^{TM} instrument in any of the experimental groups or at any sampling time.

The average value of the plasma osmolality pre-stress was 354.83 mOsm kg^{-1} (± 23.79). There were no significant differences between experimental groups and pre-stress values at 7, 14 and 21 days after the start of the experiment. At day 28 of the experiment in the stress group, the plasma osmolality was measured to 391.67 mOsm kg^{-1} (± 37.59). This was significantly higher than plasma osmolality levels from pre-stress and was significantly higher than the plasma osmolality level from the control group at day 28 (figure 2a).

The average concentration of plasma chloride at pre-stress levels were 145 mM (± 9.38). There were no significant differences between the experimental groups and pre-stress values at 7, 14 and 21 days after the start of the experiment. At day 28 of the experiment

the stress group had significantly higher plasma chloride values compared to the control group and pre-stress levels (171. 33 ± 22.44 mM) (figure 2b)

There were no significant differences in plasma magnesium levels between experimental groups and pre-stress values at 7, 14 and 21 days after the start of the experiment. At day 28 of the experiment the stress group had significantly higher plasma magnesium values compared to the control group and pre-stress levels (4.57 ± 1.62 mM) (figure 2c).

Insert Figure 2a-c

3.3 Tertiary stress responses

The average weight of lumpfish before the start of experiment was 52.7 g (± 15.1). The average weight of fish from the control group increased significantly compared to the pre-stress and daily stress groups at 21 and 28 days after the start of the experiment, and was 152.3 g (± 96.6) and 150.1 g (± 93.9), respectively (figure 3a).

Figure 3b shows the difference in average specific growth (SGR) rate between the control group and stress group at the end of the experiment (day 28). The overall SGR in the control group (2.33% ± 0.01%) was significantly higher at the end of the experiment compared to the stress group (1.27% ± 0.02%).

Insert Figure 3a-b

3.4 HPI-axis

ACTH sensitivity

The average plasma cortisol levels in stressed lumpfish injected with ACTH solution showed a significant increase in comparison to the pre-stress levels and control group after 28 days of experiment at stressed group of lumpfish 85.11 nM (± 16.05) (Figure 4a)

Negative feedback response

Figure 4b shows the average plasma cortisol levels in lumpfish injected with PBS solution and a significant increase in the daily stressed group occurred in comparison to pre-stress levels and control group after 21 and after 28 days, and was 28.98 nM (± 8.6) and 35.27 (±8.95), respectively.

Insert Figure 4a-b

4. Discussion

Production of farmed salmon and trout in Norway has increased from 360 000 tons in 1998 to 1.2 million tons in 2016 (DOF, 2017b), however the use of pesticides on farmed salmon has declined in 2016 compared to 2014 and 2015 in Norway (NIPH, 2017). This is most likely due to more alternative mechanical sea lice treatments, and the introduction of cleaner fish (Imsland et al., 2014). *C. lumpus* can be used in bigger scale and with bigger meddling percentage compared to other cleaner fish species (Chilvers, 2013). However, with the increasing use of lumpfish increasing concern for the welfare of the species has arisen as mortality and disease outbreak has started to increase in later years (Press, 2017). One possible reason to this is the increased stress load the fish experience during common aquaculture practice such as grading, cleaning, vaccination and transport (Barton, 2002; Iversen Eliassen & Finstad, 2009; Iversen et al., 2005; Iversen & Eliassen, 2014).

Pre-stress levels of cortisol in lumpfish were low and within the level that is generally considered representative for unstressed fish (Barton & Iwama, 1991). Similar resting levels of cortisol in lumpfish were found both by Iversen et al. (2015) and Haatuft (2015). Resting levels of plasma cortisol in the daily stressed lumpfish increased significantly compared to control, and reached over 20 nM at day 21 and day 28 (figure 1). This increased level of plasma cortisol does not seem to be high in comparison to experiments done on salmon and ballan wrasse. However, Iversen et al. (2015) showed that lumpfish had a different stress response to a short-term stressor compared to several other species such as ballan wrasse (*Labrus bergylta*), cod (*Gadus morhua*), and Atlantic salmon (*Salmo salar*). The lumpfish stress reaction was very similar to the reaction of Atlantic halibut (*Hippoglossus hippoglossus*) (Iversen and Eliassen, 2014). Halibut, as lumpfish, secretes moderate amounts of cortisol and tries to hide from danger. The prolonged elevated level of plasma cortisol from day 21 in the stressed fish indicates an allostatic load on the HPI axis. The stress response is a natural reaction which promotes increased survival, and thus the normal plasma cortisol surge is short lived and in most cases, should return to pre-stress levels within 12 to 24 hours after the applied stressor (Iversen & Eliassen, 2009; Iversen et al., 2005; Iversen et al., 1998; Schreck, 1982). However, long time stress can be maladaptive and malicious for fish due to changes in the sensitivity of the

HPI axis (Iversen & Eliassen, 2014; Mommsen Vijayan & Moon, 1999; Overli Pottinger Carrick Overli & Winberg, 2002; Pickering & Pottinger, 1989).

Usually stress influences levels of glucose and lactate (Iversen et al., 2003; Moon & Foster, 1995), and glucose is a commonly used indicator of secondary stress response (Cook, 2012; Mommsen et al., 1999). Earlier experiments conducted on salmonids and other species shows that the stress response often promotes increase in the glucose levels (Barcellos et al., 2001; Barton, 2000; O'Connor Pottinger & Sneddon, 2011). However, the results in the present study do not show any significant changes. Similar lack of correlation between plasma cortisol and glucose levels has been found in several other studies (Barton Schreck & Fowler, 1988; Cook, 2012; Mommsen et al., 1999). Van Heeswijk et al. (2006) mentioned that fasting the animal before the experiment influence the metabolic status of the body, and with that it can change glucose levels and stress response of the organism in question. Fasting have shown to effect ability to induce a glucose response and increase hepatic sensitivity to adrenergic stimulation (Barton et al., 1988; Van Heeswijk et al., 2006). One has also to take into consideration that plasma glucose in carnivorous fish can fluctuate a lot more than in mammals, so it may not be used as the only indicator of metabolic status or stress (Mommsen et al, 1999).

The elevation of the lactate concentrations immediately after stress is likely due to muscle glycolysis (Moon & Foster, 1995), and is the end product of glucose metabolism during anaerobe glycolysis (Olsen et al., 1995). The average concentration of lactate in blood of fish from the control group was 0.77 mM (± 0.08) before the start of experiment. However, plasma lactate was not measured above detection limit of the Lactate Pro™ instrument in any experimental groups or sampling time. This could mean that the levels of lactate were undetectably low during the experiment, and of no physiological significance. Atlantic salmon, Atlantic cod and ballan wrasse, all had elevated levels of lactate after crowding stress. These species show active fight or flight reaction during stress resulting in high muscle activity. In contrast species like Atlantic halibut and lumpfish seeks shelter or hides during sever stress, and thus, do not seem to produce any muscle lactate at all (Iversen et. al., 2015)

Release of cortisol has a direct influence on osmolality, chloride and magnesium, and one can use them as an indication of secondary stress responses (Bjornsson Stefansson & McCormick, 2011; Veiseth Fjaera Bjerkeng & Skjervold, 2006; Wendelaar Bonga, 2011; Wendelaar Bonga, 1997). The only significant difference in plasma osmolality and chloride levels from pre-stress was detected at day 28. There was no positive correlation between plasma cortisol and the elevated levels of plasma osmolality and chloride. A plausible explanation is due to a compensatory response on cell level. When fish cells are exposed to hypotonic environment the cells rapidly swell but will return to its original volume, by eliminating cellular osmolytes and hence water. This regulatory mechanism is termed regulatory volume decrease (RVD). Fish in seawater, however, will work against shrinking of cells (as an effect of elevated plasma osmolality), thus minimising the effect of the stressor (Trischitta Denaro & Faggio, 2005). However, as the stress becomes prolonged (chronic) the fish will not be able to keep up this compensatory response as all reserve energy has been depleted, thus this can explain the significant increase in both osmolality and plasma chloride in stressed group at the end of the experiment.

To prevent dehydration and because of osmotic water loss, marine teleosts must intake seawater. In the same time, they must eliminate divalent ions as magnesium and sulphate. Redding and Schreck (1983) stated that uptake of magnesium (Mg^{2+}) and excretion happens in guts and kidneys. In the most tested species of teleost total magnesium concentration in plasma will not be higher than 2nM (Bijvelds Kolar & Flik, 2001). Experiments on Atlantic cod (Staurnes Rainuzzo Sigholt & Jorgensen, 1994a), Coho salmon (*Oncorhynchus kisutch*) (Redding and Schreck, 1983), Atlantic salmon (Iversen & Eliassen, 2009; Iversen et al., 2009; Iversen & Eliassen, 2014) and gilt-head bream (*Sparus aurata* L.) (Arends Mancera Munoz Wendelaar Bonga & Flik, 1999) showed strong correlation between stress and increase in plasma magnesium concentration. The concentration of Mg^{2+} during our experiment was significantly higher at the end of the experiment in the daily stress group compared to the control group. As indicated lumpfish show abilities to cope with some secondary effects of stress in the short time perspective, however every day stress and accumulation of plasma cortisol seems to have a negative impact on the stress coping mechanism.

When stress affects the organism and population in form of growth, disease resistance and mortality, one often denotes it as a tertiary stress response (Wendelaar Bonga, 2011; Wendelaar Bonga, 1997). To establish homeostasis during and after a stressor the organism must direct all energy to important task as locomotion, and respiration. As long as the danger exists, other physical tasks as growth or reproduction will not be prioritised (Wendelaar Bonga, 2011; Wendelaar Bonga, 1997).

The average weight of lumpfish in the stressed group showed stunted growth and did not increase after 14 days. The control group, on the other hand, showed a steady rise in average weight throughout the experiment, and its weight was significantly higher than the stress group after 21 and 28 days. The average specific growth rate (SGR) shows a similar negative trend. An effect of plasma cortisol on growth has been shown at in gold fish (Bernier et al., 2004), Atlantic cod (Staurnes et al., 1994a; Staurnes Sigholt Pedersen & Rustad, 1994b) and Atlantic salmon (Pickering, 1990). In addition, Bernier (2004) noticed that, the specific growth rate was lower in the stressed group both in comparison to the control group and to the pre-stress situation, as in this lumpfish experiment.

It has been shown that during chronic stress, changes in activity and sensitivity in the HPI-axis can be recorded (Iversen and Eliassen, 2014). There are numerous methods of detecting chronic stress (allostatic overload type 2). Stimulation tests (by CRH, CRH/vasopressin, ACTH) that measure the relative sensitivity of the pituitary, and the inhibition test using dexamethasone (DEX) to show the reduced possibilities of the negative feedback (Mormede et al., 2007). The design of the stimulation test was based on Pottinger and Carrick (2001) and was used in this lumpfish experiment with minor modifications. After 28 days the daily stressed group showed oversensitivity to the weight-adjusted dose of ACTH compared to the control group. Even though studies on ACTH sensitivity in animals are not very numerous, those which has been conducted supports the notion that chronic stress development is due to changes in HPI axis activity and sensitivity (Friend Dellmeier & Gbur, 1985; Iversen & Eliassen, 2014; Mormede et al., 2007).

The inhibition test using dexamethasone showed significant changed negative feedback mechanism in lumpfish exposed to the daily stressor. Historically, the DEX test has been used to detect HPI axis changes in melancholic patients (Banki Arato Papp

Rihmer & Kovacs, 1986; Kumar Alcser Grunhaus & Greden, 1986; Wilens Ritchie & Carroll, 1984). Depressed people reacted in a smaller degree to DEX injections, compared to healthy individuals who experienced a significant reduction in the cortisol's morning peak (Banki et al., 1986; Kumar et al., 1986; Wilens et al., 1984). Similarly, to depressed people, chronically stressed animals (with allostatic overload type 2), shows the same reaction to DEX injections, and similar regularities were found in chronic stressed Atlantic salmon (Iversen and Eliassen, 2014). Already after three weeks, a significant difference occurred between the stress group and the control group and the difference increased with time. Similar results were shown in Iversen and Eliassen (2014) where salmon exposed to daily stress in four weeks became oversensitive concerning ACTH stimulation, and had a reduced negative feedback system, and thus, elevated resting levels of plasma cortisol. This most likely represented an allostatic overload type 2 response with dire consequences for the health and welfare of the individual fish. However, there are very few studies (Iversen and Eliassen, 2014) on chronic stress of fish, combining the baseline levels of plasma cortisol, sensitivity of the interrenal cells (ACTH), and the efficiency of the negative feedback by corticosteroids (DEX). Lack of similar tests and unclear definitions of acute/chronic stress makes it difficult to draw precise conclusions, and to generalize results for different fish species. However, in lumpfish (this study), Atlantic salmon (Iversen and Eliassen, 2014) and rainbow trout (Pottinger and Carrick, 2001), an oversensitive HPI-axis with reduced negative feedback system and elevated baseline levels of plasma cortisol seems to indicate a chronic stressed fish (allostatic overload type 2) with high potential to compromise the animal welfare of the species.

The results of this study indicate that lumpfish exposed to daily stress experience an allostatic overload type 2 reaction with an oversensitivity to ACTH, followed by a reduced negative feedback system with elevated baseline levels of plasma cortisol. As result, this could compromise the animal welfare. All rearing and handling of lumpfish should, therefore, be done with care and special focus on possible prolonged stressful situations, which could jeopardize the production result and the fish welfare of the lumpfish.

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References

Arends RJ, Mancera JM, Munoz JL, Wendelaar Bonga SE & Flik G (1999) The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *Journal of Endocrinology*, **163 (1)**, 149-157.

Banki CM, Arato M, Papp Z, Rihmer Z & Kovacs Z (1986) Associations among dexamethasone non-suppression and TRH-induced hormonal responses: increased specificity for melancholia. *Psychoneuroendocrinology*, **11**, 205-211.

Barcellos LJG, Woehl VM, Wassermann GF, Quevedo RM, Ittzes I & Krieger MH (2001) Plasma levels of cortisol and glucose in response to capture and tank transference in Rhamdia quelen (Quoy & Gaimard), a South American catfish. *Aquac. Res.*, **32**, 121-123.

Barton BA (2000) Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. *North American Journal of Aquaculture,* **62**, 12-18.

Barton BA (2002) Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.*, **42**, 517-525.

Barton BA & Iwama GK (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteriods. *Annual Review of Fish Diseases*, 3-26.

Barton BA, Schreck CB & Fowler LG (1988) Fasting and Diet Content Affect Stress-Induced Changes in Plasma-Glucose and Cortisol in Juvenile Chinook Salmon. *Progressive Fish-Culturist,* **50**, 16-22.

Bijvelds MJC, Kolar ZI & Flik G (2001) Electrodiffusive magnesium transport across the intestinal brush border membrane of tilapia (Oreochromis mossambicus). *European Journal of Biochemistry,* **268**, 2867-2872.

Bjornsson BT, Stefansson SO & McCormick SD (2011) Environmental endocrinology of salmon smoltification. *General and Comparative Endocrinology*, **170**, 290-298.

Brandal PO & Egidius E (1979) Treatment of salmon lice (Lepeophtheirus salmonis Krøyer, 1838) with Neguvon® — Description of method and equipment. *Aquaculture,* **18(2)**, 183-188. doi: http://dx.doi.org/110.1016/0044-8486(1079)90030-90039.

Burridge L, Weis JS, Cabello F, Pizarro J & Bostick K (2010) Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. *Aquaculture,* **306(1–4)**, 7-23.

Chilvers H (2013) Lumpfish: The latest weapon in the battle against sea lice? Hentet 02.03.2015 fra http://www.thefishsite.com/articles/1787/lumpfish-the-latest-weapon-in-the-battle-against-sea-lice/. Published 02.12, 2013.

Cook NJ (2012) Review: Minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. *Canadian Journal of Animal Science*, **92**, 227-259.

Costello MJ (2009) The global economic cost of sea lice to the salmonid farming industry. *Journal of Fish Diseases*, **32(1)**, 115-118. doi: 110.1111/j.1365-2761.2008.01011.x.

Davenport J (1985) Synopsis of biological data on the lumpsucker Cyclopterus lumpus (Linnaeus, 1958). *FAO Fisheries Synopsis,* **147**, 31 pp.

DOF (2017a) Directorate of Fisheries. Statistics for aquaculture. Cleanerfish (Lumpfish and Wrasse). Bergen, Norway, http://www.fiskeridir.no/English/Aquaculture/Statistics/Cleanerfish-Lumpfish-and-Wrasse. Updated: 29.05.2017.

DOF (2017b) Statistics for aquaculture. Atlantic salmon and rainbow trout: Sale 1994-2016. http://www.fiskeridir.no/English/Aquaculture/Statistics/Atlantic-salmon-and-rainbow-trout. Updated: 29.05.2017.

Friend TH, Dellmeier GR & Gbur EE (1985) Comparison of four methods of calf confinement. I. Physiology. *Journal of Animal Science,* **60**, 1095–1101.

Goymann W & Wingfield JC (2004) Allostatic load, social status and stress hormones: the costs of social status matter. *Animal Behaviour,* **67**, 591-602.

Haatuft AC (2015) Effects of reduced water oxygen saturation on growth and plasma cortisol levels in juvenile lumpfish (Cyclopterus lumpus L.) in aquaculture, Msc Thesis. The Arctic University of Norway, Tromsø.

Imsland AK, Reynolds P, Eliassen G, Hangstad TA, Foss A, Vikingstad E & Elvegård TA (2014) The use of lumpfish (Cyclopterus lumpus L.) to control sea lice (Lepeophtheirus salmonis Krøyer) infestations in intensively farmed Atlantic salmon (Salmo salar L.). *Aquaculture,*, **424-425**, 18-23. doi: 10.1016/j.aquaculture.2013.1012.1033.

Iversen M & Eliassen RA (2009) The Effect of AQUI-S ^{R} Sedation on Primary, Secondary, and Tertiary Stress Responses during Salmon Smolt, Salmo salar L., Transport and Transfer to Sea. *Journal of the World Aquaculture Society,* **40**, 216-225.

Iversen M, Eliassen RA & Finstad B (2009) Potential benefit of clove oil sedation on animal welfare during salmon smolt, Salmo salar L. transport and transfer to sea. *Aquac. Res.*, **40**, 233-241.

Iversen M, Finstad B, McKinley RS & Eliassen RA (2003) The efficacy of metomidate, clove oil, Aqui-S (TM) and Benzoak (R) as anaesthetics in Atlantic salmon (Salmo salar L.) smolts, and their potential stress-reducing capacity. *Aquaculture,* **221**, 549-566.

Iversen M, Finstad B, McKinley RS, Eliassen RA, Carlsen KT & Evjen T (2005) Stress responses in Atlantic salmon (Salmo salar L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. *Aquaculture,* **243**, 373-382.

Iversen M, Finstad B & Nilssen KJ (1998) Recovery from loading and transport stress in Atlantic salmon (Salmo salar L.) smolts. *Aquaculture,* **168**, 387-394.

Iversen MH & Eliassen RA (2014) The effect of allostatic load on hypothalamic-pituitary-interrenal (HPI) axis before and after secondary vaccination in Atlantic salmon postsmolts (Salmo salar L.). *Fish Physiol Biochem,* **40**, 527-538.

Iversen MH, Jakobsen R, Eliassen R & Ottesen O (2015) Sedasjon av berggylt og rognkjeks for å redusere stress og dødelighet. In: *Norsk Fiskeoppdrett,* pp. 42-46 (in Norwegian).

Juster RP, McEwen BS & Lupien SJ (2010) Allostatic load biomarkers of chronic stress and impact on health and cognition. *Neuroscience and Biobehavioral Reviews,* **35**, 2-16.

492 Kumar A, Alcser K, Grunhaus L & Greden JF (1986) Relationships of the dexamethasone
493 suppression test to clinical severity and degree of melancholia. *Biological*
494 *Psychiatry*, **21**, 436-444.
495 Liu Y & Bjelland HV (2014) Estimating costs of sea lice control strategy in Norway. *Prev*
496 *Vet Med*, **117(3-4)**, 469-477. doi: 410.1016/j.prevetmed.2014.1008.1018.
497 McEwen BS (1998) Stress, adaptation, and disease - Allostasis and allostatic load. In:
498 *Neuroimmunomodulation*, pp. 33-44.
499 McEwen BS (2005) Stressed or stressed out: What is the difference? *Journal of Psychiatry*
500 *& Neuroscience*, **30**, 315-318.
501 McEwen BS & Wingfield JC (2003) The concept of allostasis in biology and biomedicine.
502 *Hormones and Behavior*, **43**, 2-15.
503 McVicar AH (2004) Management actions in relation to the controversy about salmon lice
504 infections in fish farms as a hazard to wild salmonid populations. *Aquaculture*
505 *Research submitted.*, **35(8)**, 751-758. doi: 710.1111/j.1365-2109.2004.01097.x.
506 Mommsen TP, Vijayan MM & Moon TW (1999) Cortisol in teleosts: dynamics,
507 mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and*
508 *Fisheries*, **9**, 211-268.
509 Moon TW & Foster GD (1995) Tissue carbohydrate metabolism, gluconeogenesis and
510 hormonal and environmental influences. In: *Metabolic and Adaptational*
511 *Biochemistry, Elsevier, Amsterdam* (ed. by Hochachka PW, Mommsen TP), pp. 65–
512 100.
513 Mormede P, Andanson S, Auperin B, Beerda B, Guemene D, Malmkvist J, Manteca X,
514 Manteuffel G, Prunet P, van Reenen CG, Richard S & Veissier I (2007) Exploration
515 of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare.
516 *Physiology & Behavior*, **92**, 317-339.
517 Mozaffarian D & Rimm EB (2006) Fish intake, contaminants, and human health:
518 evaluating the risks and the benefits. *JAMA*, **296(15)**, 1885-1899. doi:
519 1810.1001/jama.1296.1815.1885.
520 NIPH (2017) Norwegian Institute of Public Health. Legemidler i fiskeoppdrett 2016. 2016:
521 Salg av lakselusmidler er synkende.
522 http://www.fhi.no/hn/legemiddelbruk/fisk/2016-salg-av-lakselusmidler-er-
523 synkende/ Published 28.02.2017 (in Norwegian).
524 Norethberg G, Johannesen A & Arge R (2015) Cryopreservation of lumpfish Cyclopterus
525 lumpus (Linnaeus, 1758) milt. *PeerJ,*, **3**, e1003. doi: 1010.7717/peerj.1003.
526 Nytrø AV, Vikingstad E, Foss A, Hangstad TA, Reynolds P, Eliassen G & Imsland AK
527 (2014) The effect of temperature and fish size on growth of juvenile lumpfish
528 (Cyclopterus lumpus L.). *Aquaculture*, **434**, 296-302. doi:
529 210.1016/j.aquaculture.2014.1007.1028.
530 O'Connor EA, Pottinger TG & Sneddon LU (2011) The effects of acute and chronic
531 hypoxia on cortisol, glucose and lactate concentrations in different populations of
532 three-spined stickleback. *Fish Physiol Biochem*, **37**, 461-469.
533 Olsen YA, Einarsdottir IE & Nilssen KJ (1995) Metomidate anaesthesia in Atlantic salmon,
534 Salmo salar, prevents plasma cortisol increase during stress. *Aquaculture*, **134**, 155-
535 168.

536 Overli O, Pottinger TG, Carrick TR, Overli E & Winberg S (2002) Differences in
537 behaviour between rainbow trout selected for high- and low-stress responsiveness.
538 *Journal of Experimental Biology*, **205**, 391-395.
539 Pickering AD (1990) Stress and the supppression of somatic growth in teleost fish.
540 *Progress in Comparative Endocrinology*, 473-479.
541 Pickering AD & Pottinger TG (1989) Stress responses and disease resistance in salmonid
542 fish: effects of chronic elevation of plasma cortisol. *Fish Physiol Biochem*, **7**, 253-
543 258.
544 Pottinger TG & Carrick TR (2001) ACTH does not mediate divergent stress responsiveness
545 in rainbow trout. *Comp. Biochem. Physiol. A-Mol. Integr. Physiol.*, **129**, 399-404.
546 Press MM (2017) Forskining.no. Rognkjeksen sliter med helsa.
547 http://forskning.no/2017/04/laksens-lusespiser-sliter/produsert-og-finansiert-
548 av/veterinaerinstituttet. Published 9.4 2017 04:00. (In Norwegian).
549 Price MH, Proboszcz SL, Routledge RD, Gottesfeld AS, Orr C & Reynolds JD (2011) Sea
550 louse infection of juvenile sockeye salmon in relation to marine salmon farms on
551 Canada's west coast. *PLoS One*, **6(2)**, e16851. doi:
552 16810.11371/journal.pone.0016851.
553 Redding MJ & Schreck CB (1983) Influence of Ambient Salinity on Osmoregulation and
554 Cortisol Concentration in Yearling Coho Salmon during Stress. *Transactions of the*
555 *American Fisheries Society*, **112**, 800–807.
556 Schreck CB (1982) Stress and Rearing of Salmonids. *Aquaculture*, **28**, 241-249.
557 Selye H (1950) Stress and the general adaption syndrome. *British Medical Journal*, **1**
558 **(4667)**, 1383-1392.
559 Selye H (1973) Homeostasis and heterostasis. *Perspectives of Biological Medicine*, **16**,
560 441-445.
561 Sokal RR & Rohlf FJ (1987) Introduction to biostatistics. Freeman, New York, pp. 178-
562 179.
563 Staurnes M, Rainuzzo JR, Sigholt T & Jorgensen L (1994a) Acclimation of Atlantic Cod
564 (Gadus-Morhua) to Cold-Water - Stress-Response, Osmoregulation, Gill Lipid-
565 Composition and Gill Na-K-Atpase Activity. *Comparative Biochemistry and*
566 *Physiology a-Physiology*, **109**, 413-421.
567 Staurnes M, Sigholt T, Pedersen HP & Rustad T (1994b) Physiological-Effects of
568 Simulated High-Density Transport of Atlantic Cod (Gadus-Morhua). *Aquaculture*,
569 **119**, 381-391.
570 Taranger GL, Karlsen O, Bannister RJ, Glover KA, Husa V, Karlsbakk E & Svasand T
571 (2014) Risk assessment of the environmental impact of Norwegian Atlantic salmon
572 farming. *ICES Journal of Marine Science*, **72(3)**, 997-1021. doi:
573 1010.1093/icesjms/fsu1132.
574 Torrissen O, Olsen RE, Toresen R, Hemre GI, Tacon AGJ, Asche F & Lall S (20111)
575 Atlantic Salmon (*Salmo salar*): The "Super-Chicken" of the Sea? . *Reviews in*
576 *Fisheries Science,*, **19(3)**, 257-278. doi: 210.1080/10641262.10642011.10597890.
577 Treasurer JW (2002) A review of potential pathogens of sea lice and the application of
578 cleaner fish in biological control. *Pest Manag Sci*, **58(6)**, 546-558. doi:
579 510.1002/ps.1509.

Trischitta F, Denaro MG & Faggio C (2005) Cell volume regulation following hypotonic stress in the intestine of the eel, Anguilla anguilla, is Ca2+-dependent. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, **140**, 359-367.

Van Heeswijk JCF, Vianen GJ & van den Thillart GEEJM (2006) The adrenergic control of hepatic glucose and FFA metabolism in rainbow trout (Oncorhynchus mykiss): Increased sensitivity to adrenergic stimulation with fasting. *General and Comparative Endocrinology,* **145**, 51-61.

Varsamos S, Flik G, Pepin JF, Bonga SEW & Breuil G (2006) Husbandry stress during early life stages affects the stress response and health status of juvenile sea bass, Dicentrarchus labrax. *Fish & Shellfish Immunology,* **20**, 83-96.

Veiseth E, Fjaera SO, Bjerkeng B & Skjervold PO (2006) Accelerated recovery of Atlantic salmon (Salmo salar) from effects of crowding by swimming. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology,* **144 (3)**, 351-358.

Wells RMG & Pankhurst NW (1999) Evaluation of simple instruments for the measurement of blood glucose and lactate, and plasma protein as stress indicators in fish. *Journal of the World Aquaculture Society,* **2**, 276-284.

Wendelaar Bonga SE (2011) Hormonal responses to stress. In: *Encyclopedia of Fish Physiology* (ed by Anthony PF). Academic Press, San Diego, pp. 1515-1523.

Wendelaar Bonga SEW (1997) The stress response in fish. *Physiological Reviews,* **77**, 591-625.

Wilens TE, Ritchie JC & Carroll BJ (1984) Comparison of plasma cortisol and corticosterone in the dexamethasone suppression test for melancholia. *Psychoneuroendocrinology,* **9**, 45-55.

Wingfield JC (2005) The concept of allostasis: Coping with a capricious environment. *Journal of Mammalogy,* **86**, 248-254.

608 **Figure legends**

609

610 **Figure 1**. The average values of resting levels of plasma cortisol (n ± SD nM) in the control
611 group (filled circle) and daily stress group (filled triangle) in lumpfish during 28 days of
612 exposure (n=6). # indicates significant difference between groups at the same sampling day
613 at 95% confidence level, * indicates significant difference from the pre-stress level within
614 the same group at 95% confidence level.

615

616 **Figure 2. a.** The average values of osmolality (n ± SD mOsm kg^{-1}) in the control group
617 (filled circle) and daily stress group (filled triangle) in lumpfish during 28 days of exposure
618 (n=6). **b.** The average values of plasma chloride (n ± SD mM) in the control group (filled
619 circle) and daily stress group (filled triangle) in lumpfish during 28 days of exposure (n=6).
620 **c.** The average values of plasma magnesium (n ± SD mM) in the control group (filled
621 circle) and daily stress group (filled triangle) in lumpfish during 28 days of exposure (n=6).
622 # indicates significant difference between groups at the same sampling day at 95%
623 confidence level, * indicates significant difference from the pre-stress level within the same
624 group at 95% confidence level.

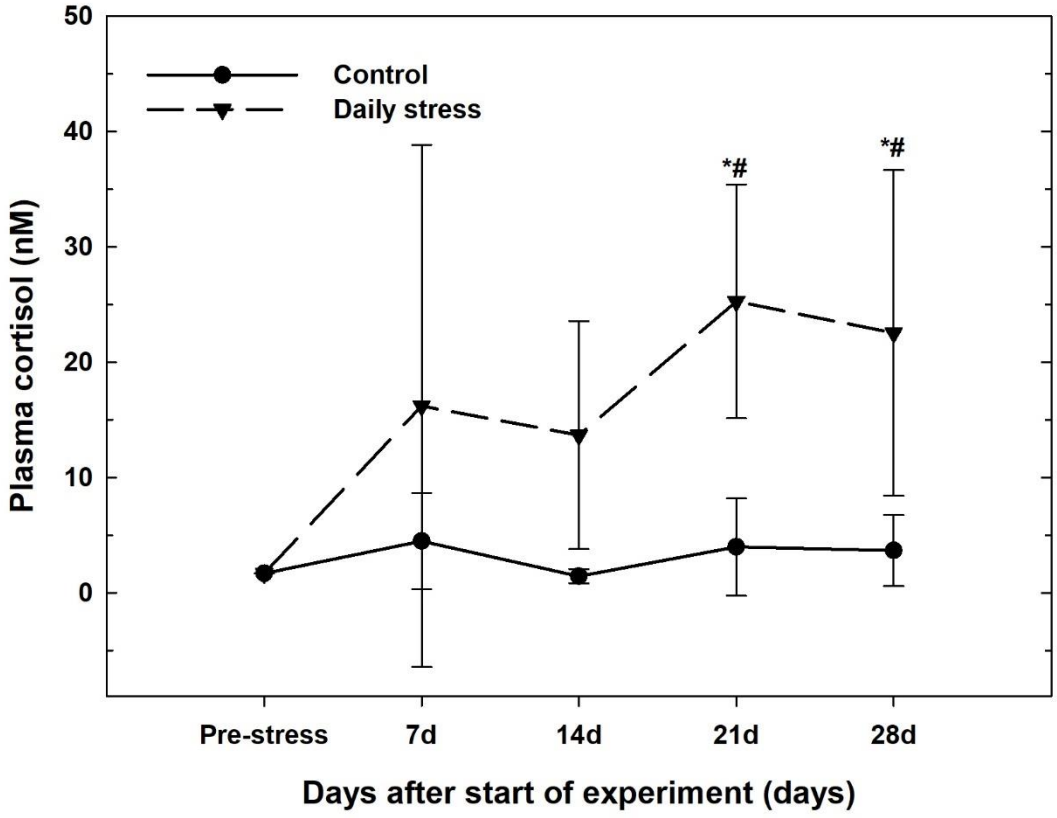
625

626 **Figure 3. a.** The average weight (g) (n ± SD mOsm kg^{-1}) in the control group (filled circle)
627 and daily stress group (filled triangle) in lumpfish during 28 days of exposure (n=18). **b**.
628 The overall specific growth rate (%) at the end of experiment (from day 0-28) in the control
629 group (black bars) and daily stress group (gray bars) (n=18). # indicates significant
630 difference between groups at the same sampling day at 95% confidence level, * indicates
631 significant difference from the pre-stress level within the same group at 95% confidence
632 level.

633

634 **Figure 4.** Average plasma cortisol levels in the control group (black bars) and daily stress
635 group (gray bars) in lumpfish during 28 days of exposure (n=12) following intraperitoneal
636 injection with either **a.** adrenocorticotropic hormone (ACTH 45 µg mL^{-1}, 0.5 mL kg^{-1}) or **b.**
637 negative sensitivity test; phosphate buffered saline (PBS; 0.5 mL kg^{-1}). All fish were

injected 24 h previously with dexamethasone (DEX; 1 mg kg^{-1} in ethanol: PBS; 1:3; 1 µg L^{-1}). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.



642

643 **Figure 1.**

644

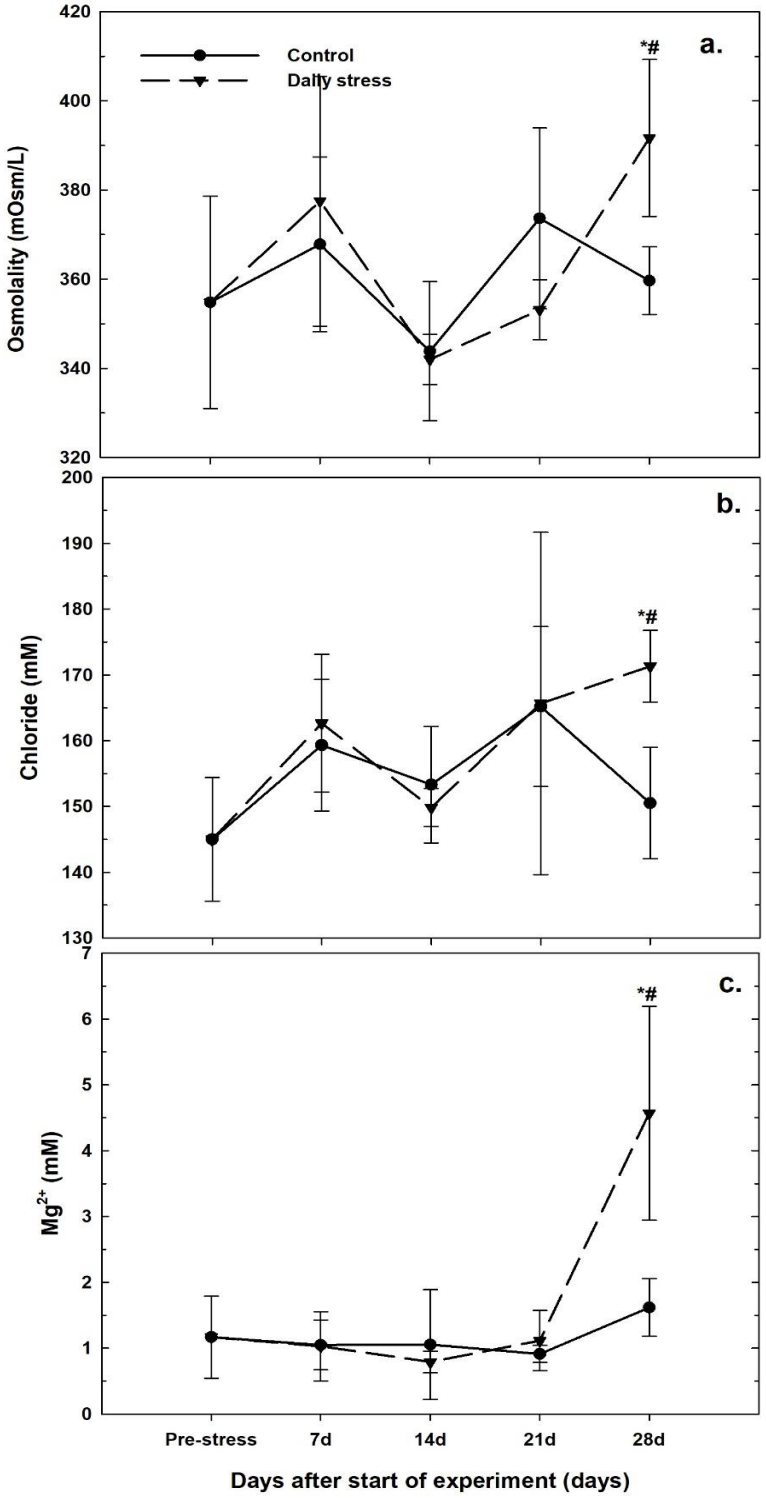

a.
Control
Daily stress
*#
Osmolality (mOsm/L)
420
400
380
360
340
320
b.
*#
Chloride (mM)
200
190
180
170
160
150
140
130
c.
*#
Mg2+ (mM)
7
6
5
4
3
2
1
0
Pre-stress
7d
14d
21d
28d
Days after start of experiment (days)

Figure 2a-c.

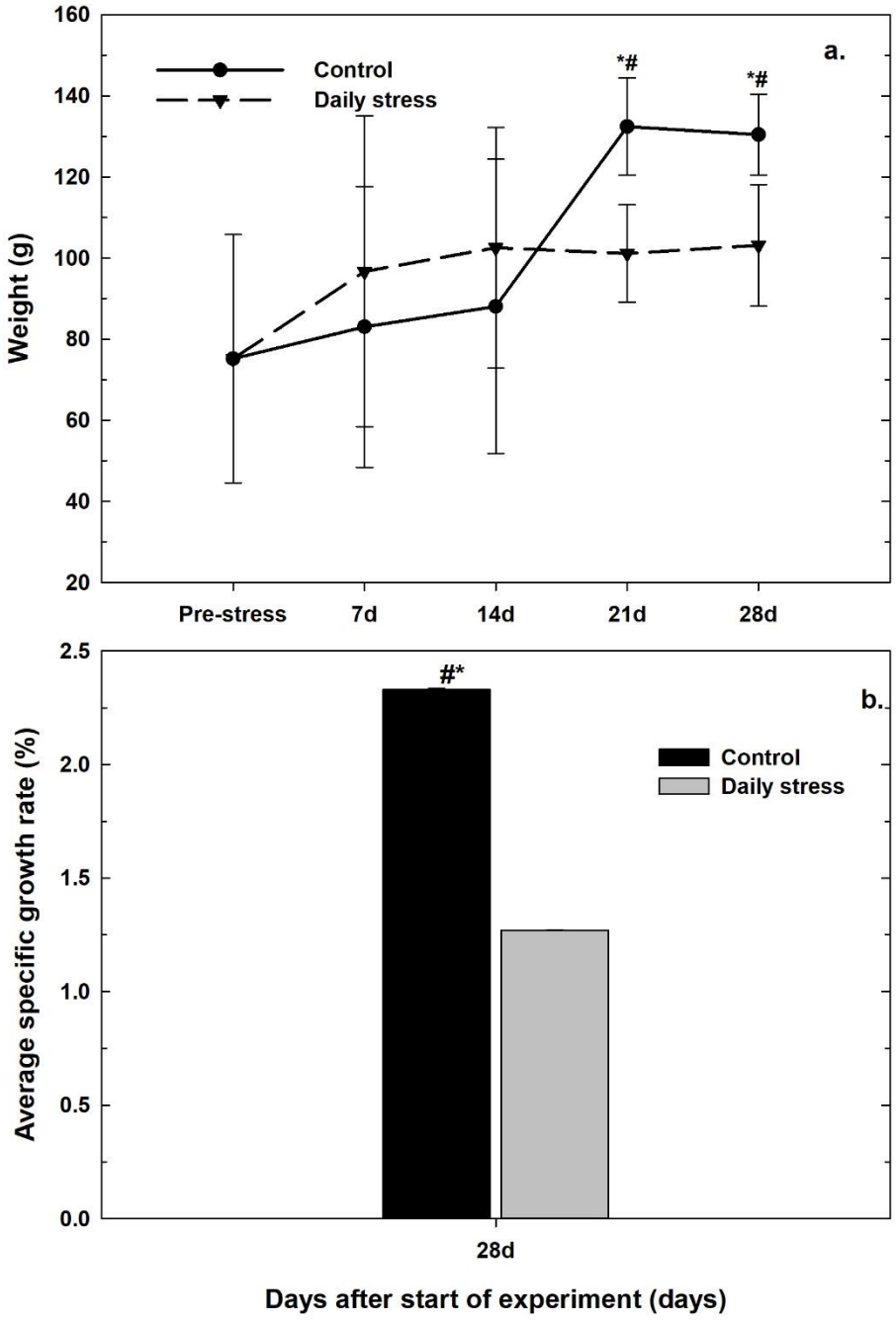
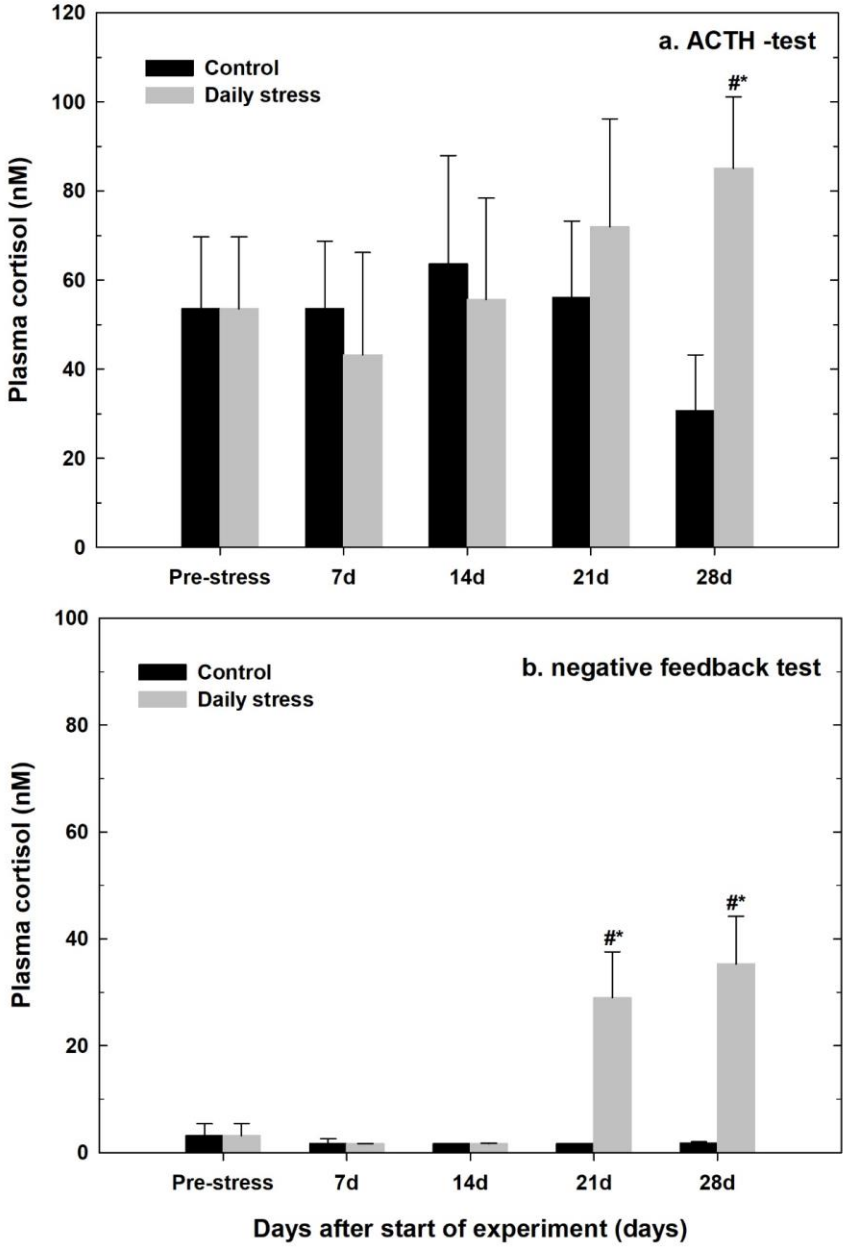

a.
Control
Daily stress
*#
*#
Weight (g)
160
140
120
100
80
60
40
20
Pre-stress
7d
14d
21d
28d
b.
#*
Control
Daily stress
Average specific growth rate (%)
2.5
2.0
1.5
1.0
0.5
0.0
28d
Days after start of experiment (days)

Figure 3a-b.



650

651 **Figure 4a-b.**

Paper V

A multi-omics approach to assess the effect of temperature acclimation on lumpfish (*Cyclopterus lumpus*) skin

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Abstract

Fish live in an environment subjected to temperature variations on daily and seasonal basis. The role of genes and proteins in acclimation to varying temperature is poorly understood in lumpfish. We used 2D gels coupled with tandem mass spectrometry and RNA sequencing to examine the changes in protein and gene expression in lumpfish skin raised on 8 °C , then at 14 °C (warm) and 8 °C (cold) for 7 days before sampling. The differentially expressed proteins were small ubiquitin-related modifier 2, prefoldin subunit 5, SH3 domain-binding glutamic acid-rich-like protein, protein mago nashi homolog, proteasome subunit beta type 1-A, retinoblastoma binding protein 4-like, angiotensin-like, microfibril-associated glycoprotein 4-like, apolipoprotein E, 40S ribosomal protein, annexin A3 and catechol methyltransferase domain- containing protein 1-like protein. RNA seq analysis found the genes; thioredoxin interacting protein, phosphate and actin regulator, disintegrin and metallopeptidas with thrombospondin motif, metallopeptidase with thrombospondin type-1, proline synthase co-transcribed homolog, atherin and catenin delta 2 to be differentially expressed. Our protein expression data showed 17 proteins spots with 1.5 fold change, but the gene expression data showed fold changes (<1) between the warm and cold temperature acclimated fish. The result from the present study indicates that lumpfish skin, an important mucosal surface shows moderate changes when kept at 14 °C compared with 8 °C.

Introduction

Temperature is one of the important environmental parameters in fish farming. Fishes are ectothermic (exception: tuna (genus *Thunnus*) animals that do not produce constant heat to regulate their body temperature like human [1, 2]. They depend on the surrounding water temperature to control their body temperature [1]. Despite being ectothermic and having the ability to adapt to a wide range of temperature, changes in temperature beyond the tolerance limits may have bad impact on fish growth and survival [3].

Thermal tolerance in fish changes between species, age, temperature of acclimation and the duration of exposure [4-6]. Variation in temperature initiates several changes at the molecular level such as change in expressions of certain genes or proteins that are involved in metabolic pathways, cell cycle, protein folding, protein or DNA repair, and removal of toxic substances accumulated due to thermal stress [7-10].

Lumpfish (*Cyclopterus lumpus*) are widely used as a biological and environmental friendly method for removal of sea lice in Atlantic salmon farming [11]. It is a marine cold-water fish from the family Cyclopteridae. The optimal farming conditions for successful and robust lumpfish production are under investigation.

Lumpfish has been a suitable species for delousing in cold waters compared to ballan wrasse (*Labrus bergylta*), and other cleaner fish used for salmon farming. A study by Nytrø et al., 2014 showed that the ambient temperature for lumpfish growth increases with decreasing fish weight [12]. However, few studies has been done on thermal biology of lumpfish. Taking into consideration that temperature influences the physiology, biochemistry, metabolism and growth of fish [3], this study aims to find the effect of temperature acclimation on skin of lumpfish.

Here we report, the effect of temperature on theskin of lumpfish kept under two different temperatures (8 °C and 14 °C). We used 2D gel based proteomics and RNA sequencing (RNA-seq) methods to assess the molecular changes induced by temperature. The present study gives the first insights into temperature acclimation of lumpfish skin at the molecular level.

Materials and methods

Fish rearing and experimental set up

Hatchery reared lumpsucker was brought to Mørkvedbukta Research Station, (WGS84: 67°16'41.7"N 14°33'26.8"E) Nord University, Norway from Arctic Cleanerfish in Stamsund, Lofoten (WGS84: 68°7'5.0"N 13°47'14.2"E). The fish was kept at a water temperature of 10-12 °C for 60 days and then at 7-8 °C until use. They were fed with the commercial feed Amber Neptun 3 mm. Throughout the experiment period the oxygen content in the water was maintained at 80 % and the photoperiod was 24L:0D. The acclimation was done in four circular grey fiber-glass tanks, each with a volume of 400 l with a water flow of 150 l/hour. A total of 80 lumpsuckers were randomly allocated from holding tanks to the four-acclimation tanks (20 fish/ tank). Two of the acclimation tanks were held at a temperature of approximately 8 °C (7.6 ±0.1 °C), while the other two received additional heated seawater from a main tank bring the temperature to 14 °C (13.7 ±0.2 °C). The temperature in the warm acclimation tanks were increased just after lumpsuckers were transferred to the tank and reached a final temperature of 14 °C in a span of 48 h. The temperature acclimation was continued until day 7 after temperature started to increase. 6 fish from each tank was sampled from the two cold acclimated tanks and the two warm acclimated tanks, a total of 12 fish from each temperature were sampled. This study was conducted in accordance with The Norwegian Regulation on Animal Experimentation (FOR-1996-01-15-23) and the Animal Welfare Act (LOV-2009-06-19-97), FDU application number 7835.

Skin sampling

Prior to skin collection, the fishes were anaesthetized using MS-222 (300 mg/l) and killed by a blow to the head. For proteomics work skin samples (n=12, 6 each from cold and warm water acclimated fish)were snap frozen in liquid nitrogen and stored at -80 °C for later use. For RNA sequencing the skin samples (n=12, 6 from each cold and warm water acclimated tanks, samples collected from all 4 tanks) were put in 2 ml tubes containing QIAzol lysis reagent (Qiagen, Hilden, Germany) and zirconium beads (2.8 μm) and kept on

ice till the sampling for all fishes were finished. Immediately after sampling, the samples were homogenized and RNA was extracted.

Proteomic analysis

Protein extraction and 2D analysis

Frozen skin samples were homogenized with liquid nitrogen by using a pestle and mortar, mixed with 1x PBS containing protease inhibitor (GE Healthcare Life Sciences) and sonicated two times (5 s each with an interval of 1 min) on ice using an ultrasonic processor (SONICS Vibracell VCX750, USA). The sonicated sample was centrifuged at 15,000g for 30 min at 4 °C to pellet debris. The supernatant was processed as described in [12]. Protein was quantified using a Qubit Fluorometer (Invitrogen, USA). IPG strips (pH 3-10, 17cm, BioRad) were rehydrated with 100 µg of protein and electrofocused. Electrofocused strips were reduced and alkylate in SDS buffer, run on 12.5% polyacrylamide gels for approximately 16 h and stained in SYPRO Ruby (Thermo Fisher, USA)fluorescent protein stain. Gel images were documented using ChemiDocTM XRS system (BioRad, USA) and used for PD Quest Advance analysis (BioRad, USA).

LC-MS/MS and protein identification

Differentially expressed spots were excised and subjected for LC-MS/MS analysis. LC-MS/MS analysis was performed by using nanoAcquity ultra performance liquid chromatography and Q-TOF mass spectrophotometer (Micromass/Waters, MA, USA). The peak list files generated were analysed by MASCOT MS/MS Ion search (version 2.5). A homology driven search was performed using against the protein databases SwissProt (556196 sequences; 199341870 residues, 20th Dec 2017) and NCBInr (135744157 sequences; 49805139192 residues, 20th Dec 2017) for protein identification. Parameters set for identification were carbamidomethyl (C) fixed modification, oxidation (M) variable modification, monoisotopic, peptide charge 2+ and 3+, enzyme trypsin with maximum 1 missed cleavage, peptide tolerance 100 ppm and MS/MS tolerance 0.1 Da. The identification was restricted to the taxonomic group Actinopterigyii keeping the false discovery rate below 1 %. Proteins showing significant hits ($p<0.05$) with a score above

threshold level and at least one unique peptide sequence were identified. The gene ontology terms (biological process) for expressed proteins were retrieved from UniProt database.

Transcriptomic analysis

RNA extraction

The total RNA extraction was carried out immediately after tissue sampling using QIAzol lysis reagent (Qiagen, Hilden, Germany) following the manufacturer's protocol with the modification that the samples were incubated in isopropanol for 2 h instead of 10 min as described in the original protocol. The RNA integrity was assessed by RNA screen tape (Agilent, USA) using Agilent tapestation 2200 instrument (Agilent, Santa Clara, USA).

Library preparation and sequencing

mRNA libraries (n=24, 12 each from cold and warm water acclimated fish, libraries were produced from individual fish) were prepared using non directional NEBNext ultra RNA library prep kit (New England Biolabs, UK) following the manufacturer's protocol with few modification. Isolation of mRNA from the total RNA sample (1µg input) was performed using NEBNext Poly(A) mRNA magnetic isolation module (New England Biolabs, UK). The modifications were as follows, (i) for PCR enrichment of adaptor ligated DNA; 11 PCR cycles were performed to avoid over amplification, (ii) for size selection of adaptor ligated DNA; only 1st bead selection was performed with 50 µl of resuspended AMPure XP Beads to 100 µl ligation reaction. All 24 libraries were normalized and pooled before sequencing. Sequencing was performed as 150 bp paired end reads on Illumina NextSeq instrument.

Data analysis

We used cutadapt to remove low quality sequences (Phred quality score < 30) and adapter sequences. Reads with length below 50 bp were removed. The trimmed sequences were checked for the quality using FastQC. The *de novo* assembly was performed using Trinity *de novo* assembler. The quality of the assembly was assessed using Bowtie2 aligner. The assembly was used as reference for down-stream analysis. Transcript abundance was

estimated using RSEM [14]. The counts obtained from RSEM were used for differential gene analysis. The differential expression of genes was assessed using DESeq2 (log fold change of 1.5, padj <0.05 and p-value < 0.05). The statistically significant gene sequences were blasted and mapped against Actinopterygian database downloaded from UniProt KB. The gene ontology terms (GO terms) of expressed genes were retrieved using Blast2GO.

Results

Proteomic analysis

We identified 17 differentially expressed proteins using MASCOT (Fig 1a and 1b). The excised spots are highlighted in Fig 2 and protein identification details are mentioned in Table 1. Among 17 proteins, 5 did not show any significant hit in MASCOT. The identified proteins are small ubiquitin-related modifier 2, prefoldin subunit 5, SH3 domain-binding glutamic acid-rich-like protein, protein mago nashi homolog, proteasome subunit beta type 1-A, retinoblastoma binding protein 4-like[SEP], angiotensin-like, microfibril-associated glycoprotein 4-like, apolipoprotein E, 40S ribosomal protein, annexin A3 and catechol methyltransferase domain- containing protein 1-like protein. The GO-terms were retrieved from UniProt database from its human orthologs (Table 1).

De novo transcriptome assembly

Skin transcriptome of lumpsucker was *de novo* assembled using 24 sequenced libraries (12 from each cold and warm acclimation). Trinity assembled a total of 313712 transcripts and 213159 genes with an average transcript length of 1346.15 and a GC content of 46.93%. Trinity assembly statistics are mentioned in Table 2. To assess the quality of the assembly we used Bowtie2 aligner that showed 99.70% alignment rate.

Differential gene expression

The differentially expressed genes between the warm and cold temperature acclimation were analysed with DESeq2 program. A total of 28 genes showed significant difference in expression level (Fig.4). The identified genes were thioredoxin interacting protein (2

genes), phosphate and actin regulator (2 genes), disintegrin and metallopeptidas with thrombospondin motifs (7 genes), metallopeptidase with thrombospondin type 1 (1 gene), proline synthase co-transcribed homolog (3 genes), atherin (3 genes), CTND2 fragment (2 genes), catenin delta 2 (1 gene), no significant blast hit (2 genes)(Table 3).

Discussion

The results of this study showed a number of proteins and genes expressed in the lumpfish skin due to cold and warm temperature acclimation. The proteomics approach identified 17 differentially expressed proteins, of which 5 did not get any significant hits. The RNA-seq identified 28 genes to be significantly expressed in the warm temperature acclimated group compared to the cold-water group, of which 2 genes did not get any blast hits. Annotation becomes difficult with non-model species like lumpfish that have no sequences in the public databases.

Proteins and genes identified in the present study may be involved in various pathways related to temperature acclimation. A RNA-seq study on channel catfish expressed some of the molecules in gill and liver exposed to heat stress [15] that were also differentially expressed in our lumpfish study such as proteasome subunit beta type-1(involved in protein degradation), protein mago nashi homolog (involved in protein biosynthesis) and A disintegrin and metalloproteinase with thrombospondin motifs (involved in cytoskeletal organization). The proteasome subunit beta type 1 and protein mago nashi were downregulated in the heat stressed catfish group compared to control [15]. Similar results were also obtained in our study. Some of the identified molecules in our study such as angiotensinogen, 40S ribosomal protein SA, apolipoproteinE, catenin, were found in the skin mucus of chronically stressed gilthead sea bream (*Sparus aurata*) [16].

Differentially expressed proteins/genes

We have grouped the proteins/genes in 5 groups based on available literature such as protein folding and degradation, protein biosynthesis, energy metabolism, cytoskeletal organization, cell proliferation and apoptosis [10, 14, 17].

Protein folding and degradation

Small ubiquitin related modifier (SUMO) proteins are involved in sumoylation pathway that is an important protein post-translational modification. It is involved in many cellular processes such as transcriptional regulators, RNA binding and ubiquitin conjugation [18]. The gene ontology biological process shows that SUMO-2 is involved in protein sumoylation (GO:0016925) and positive regulation of proteasomal ubiquitin dependent protein catabolic process (GO:0032436). This protein has not been observed in thermal acclimation studies in teleosts but has been found to be involved in basal thermotolerance in Arabidopsis (*Arabidopsis thaliana*) [19].

Proteasome subunit beta type 1-A (PSMB1) is a subunit of proteasome that is present in the cytoplasm and it is a major protein degradation site in cells. Information about proteasome subunit beta type, in fish is scarce, however proteasome such as alpha subunit has been observed in the mucus of naïve Atlantic cod [20] and proteasome 26S subunit levels are significantly upregulated after the infection Atlantic cod with *V. anguillarum* [21]. In a study by Lu et al., 2015 the annual fish (*Nothobranchius rachovii*) were exposed to three temperatures: above (30 °C) and below (20 °C) and the normal (25 °C) for 45 days where low temperature induced high 20S proteasome activity and high temperature inhibited 20S proteasome activity. Thus, the authors suggested that cellular degradation activity during low temperature acclimation might extend the lifespan [22]. In the current lumpfish study similar observation in the skin were noticed where PSMB1 showed comparatively high expression in low temperature than in warmer temperature and this indicate that the ATP-dependent proteolysis way was repressed in order to acclimatize to the temperature change and similar observation were found in channel catfish upon heat stress [15].

Prefoldin subunit 5 (PFDN5) is a cytosolic co-chaperone that binds specifically to cytosolic nascent polypeptides and promotes folding. It is a highly specialized co-chaperone for actin and tubulin folding. It mediates transfer of newly synthesized proteins from heat shock protein complexes to the cytosolic chaperonin [23]. Prefoldin is composed of six different subunits with molecular masses in the range 14–23 kDa [24]. The protein spot we identified in the present study showed a molecular mass of 14 kDa and spot intensity was reduced in warm acclimation compared to cold (Fig 1a).

SUMO-2 and PSMB1 are involved in ubiquitin/proteasome pathway that is the major destination for unfolded or damaged proteins that are not salvaged through chaperone stabilization or refolding. In the study these proteins spots are less expressed in the warm acclimated fishes this could mean that less of these proteins are present, but could also be due to lower expression of one specific isoform. We cannot know from our data whether these spots represent active or inactive isoforms.

Protein biosynthesis

SH3 domain-binding glutamic acid-rich-like protein (SH3BGRL) belongs to thioredoxin-like protein family that are involved in antioxidant activity [25]. The gene expression profiles of *Trematomus bernacchii* in a 7 day multi-stressor treatment showed upregulation of redox protein SH3BGRL3 [26]. In the present study, it was downregulated in warm acclimation. The mechanism behind this is not clear. The oxygen saturation in the water throughout the experiment period was same, however increased temperature increases oxygen use in the body. Oxidative stress may alter the pH or give deviation in acid-base and impact the redox potential in the cell tissues. The acute increases in temperature results in transient oxidative stress and changes in antioxidant enzyme activities, which has been demonstrated in several fish species [27, 28].

Protein mago nashi homolog (MAGOH) is involved in splicing, transport of mRNAs and deciding mRNA subcellular location [29]. Mago nashi homolog is involved in transcription and translational processes. In a RNAseq study of catfish subjected to temperature stress for 4 days showed downregulation of protein mago nashi transcripts both in liver and gills [15] as it was seen in our lumpfish study.

Retinoblastoma binding protein 4, like (RBBP4) regulates chromatin metabolism, promote histone deacetylation and transcriptional repression. Another RBBP protein subunit, RBBP2 acts as an antisense protein for the hypoxic condition in euryoxic fish (*Gillichthys mirabilis*), upregulation of RBPP2 was observed strongly after 48 h in liver and skeletal muscle [30]. Histone-binding protein RBBP4 was upregulated in the muscle regeneration in trout [31]. The RBBP4 was involved in the protein synthesis and translation according to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway and upregulation of this

protein was observed in zebrafish challenged with acute metal exposures [32]. In the present study, the upregulation in acclimatised fishes might be controlling the cell proliferation in the skin.

Angiotensinogen-like (AGT) is the precursor of angiotensin. Renin-angiotensin (RAS) plays a role in regulating blood and fluid balance. Moreover, recent research also revealed oxidative stress as one of the cause for hypertension [33]. Angiotensin-1 converting enzyme (ACE) is involved in regulation of blood pressure via renin-angiotensin and the kinin- kallikrein systems [34]. In the salmon, upregulation of angiotensinogen was observed when injected with cortisol [35]. AGT was also observed in the chronically stressed skin mucus of gilthead seabream (*Sparus aurata*) which were involved in acute phase response signaling pathway [16]. In the current study angiotensinogen expression was high in warm acclimation that might be due to elevated blood pressure in the fishes and this protein might be able to reduce the high blood pressure. Here, we speculate that the exposure of lumpsucker to warm acclimation could impair cardiovascular functions and decrease circulatory oxygen concentration, thus it may induce tissue hypoxia despite ample oxygen supply from the environment. In the experiment oxygen was kept above 80 % throughout the acclimation period and hypoxia-induced pathways might be supressed by cold acclimated stress. However, it remains unclear whether fishes were hypoxic or not and whether they can develop a resistance to hypoxia or not.

40S ribosomal protein (RPS) is a protein present in the cellular organelle ribosomes responsible for protein synthesis in cells. After warm acclimation, the ribosomal protein was upregulated. Ribosomal proteins are significantly upregulated in Arctic charr exposed to sub-lethal heat stress that exhibited tolerance to acute [36]. Ribosomal proteins have been recognised as biomarker for cellular stress [37] and indicating that biogenesis occurred to the heat exposure [36]. Ribosomal proteins are involved in three important pathways, EIF2 signaling, mTOR signalling and regulation of eif4 and p70s6k signalling and was observed in chronic stressed gilthead seabream in interconnected canonical pathway [16]. 40S ribosomes binding protein in the current study might be involved in the acute response, by activating p53 pathway that plays an essential role in monitoring the balance between cellular growth and proliferation.

Energy metabolism

Apolipoprotein E (APO-E) is a lipid binding protein and a transporter of cholesterol that participates in the regulation of plasma cholesterol and lipid metabolism. The major physiological role of APO-E consists of mediating the cellular recognition and internalization of lipoproteins with members of the low-density lipoprotein receptor superfamily [38, 39]. APO-E upregulation was reported in the cold tolerant (0.7±0.05 °C) fishes when exposed to 18 h of cold stress in olive flounders (*Paralichthys olivaceus*) and the expression was declined in the cold sensitive fishes [40]. Upregulation of apolipoprotein was also observed in zebrafish [41] to a cold stress. Apolipoprotein B (APO-B), apolipoprotein C (APO-C) and apolipoprotein E (APO-E) were found up-regulated in liver of spotted sea bass in high salinity group compared to the control group [42]. The role of apolipoprotein is not only for the lipid homeostasis but equally important for their antibacterial activity for microorganisms in the mucus of the fish [43]. Thus, in the current study upregulation of APO-E in short-term warm acclimation could be due to lipid membrane alteration in the skin and the cholesterol transport might increase in warm temperature acclimated lumpfish skin to stabilize lipid fluidity by the enrichment of cholesterol in cell membranes.

Catechol O-methyltransferase (COMT) domain-containing protein 1-like (COMTD1) is involved in the O-methyltransferase activity and its function in the skin of fishes is not established yet. The COMTD1 observed in the zebra fish larvae was deceased in response to a steroid treatment (1α,25(OH)2D3) for 7 days [44]. In the crimson spotted rainbowfish, COMTD1 was downregulated in the liver of fish acclimated at a temperature of 33 °C for 14 days [45]. In higher animals, it is present in soluble and membrane-bound forms. COMT is known to be have high expression levels salinity in crustacean when exposed to low salinity [46].

Cytoskeletal organisation

Microfibril-associated glycoprotein 4-like (MFAP4) is a fibrinogen related protein. MFAP4 is expressed in the acute phase related to the innate immune response and is in a family of proteins containing FBG (C-terminal fibrinogen) domains that differ in N-terminal [47].

MFAP family members are found universally in vertebrates and invertebrates, in humans observed in extracellular matrix. MFAP4 gene expressed in brain, gill, head kidney, heart, liver, stomach, intestine, spleen, trunk kidney, skin and muscle of Mediterranean mussel (*Mytilus galliprovincialis*) [47]. In the present study, upregulation of this protein might contribute to repair or modification of tissues during temperature acclimation. Further studies are needed to establish the exact role of MFAP4 in the stress condition of fish.

A disintegrin and metalloproteinase with thrombospondin motif 8 (ADAMTS) and metallopeptidase with thrombospondin motifs are extracellular enzymes. ADAMTS have a compound domain structure. As the name suggests it has a disintegrin like-domain, a metalloproteinase domain, a thrombospondin motif, and a cysteine rich domain [52]. A study conducted to find antitumor activity of ADAMTS8 showed that it can suppress the tumour formation by disassembling the actin stress fibres in the tumour cells but not in control cells [53], that may suggests its role in cytoskeletal reorganization upon any stressful event. This gene was significantly upregulated in gill and liver of catfish in response to heat stress [15]. In goby (*Gilichthys mirabilis*) several contigs were identified as ADAMTS (with a 2 fold change) in the gill tissue of fishes that were exposed to heat stress compared to control group [54]. Expression of this gene due to temperature change might be to involve in cytoskeletal reorganization.

Phosphatase and actin regulator 1 (PHACTR1) is known for its role in actin filament rearrangement activity [55]. In a study where the PHACTR1 gene was knocked down accumulation of stress fibres and a check on cell migration was observed compared to the control, that happened because of the absence of actin polymerization and depolymerisation that was led by PHACTR1 [56].

Delta catenins are involved in cell-cell junctions. Catenin delta 2 belongs to the beta catenin superfamily of proteins that could bind to cadherin and promote actin cytoskeleton organization by modulating cell adhesion and process elaboration [57, 58]. Expression of cytoskeletal genes in skin due to thermal acclimation has been seen in many studies in fish.

Most of the genes expressed in the RNA-seq analysis of lumpfish skin in the current study were involved in cytoskeletal organization. This might be because of temperature change on skin in the beginning of the experiment, as skin disintegration might happen due to

temperature change. However, the low expression might suggest that in the beginning of the experiment the fish might have to express these genes for cytoskeletal reorganization (affected by temperature), but as the time passed it might regained its homeostasis and got acclimatized with the new conditions.

Cell proliferation and apoptosis

Annexin A3 (ANXA3) belongs to large calcium dependent protein family connected to cell membrane phospholipids that are basically involved in cellular functions, like membrane trafficking, exocytosis, endocytosis, membrane-cytoskeleton interactions, anti-inflammatory activities, signal transduction and regulation of membrane protein activities [48-51]. In the present study annexin A3 in the skin was upregulated in the warm acclimation temperature.

Other genes

Thioredoxin interacting (TXNIP) gene encodes the thioredoxin interacting protein that is an important factor of thioredoxin system. It is a negative regulator of thioredoxin function that can supress the thioredoxin activity resulting in oxidative stress [59]. TXNIP was differentially expressed in clam (*Corbicula fluminea*) raised in 26°C compared to control raised in 20°C for long term but it did not show any changes in expression under acute thermal stress [60].

In the current study we identified proteins and genes that may have direct or indirect involvement in immune/stress related pathways, but the expression levels were very low. Therefore, we assume that the lumpfish were capable of coping with the cold and warm temperature. The proteins and genes expressed in the present study are more an indicator of thermal tolerance to regain homeostasis than a thermal stress that impairs growth and survival. Thus, result from this study indicates that lumpfish can thrive well in both 8°C and 14°C temperature. This was supported by the analysis of sample distances where the two temperature groups did not separate from each other (Fig 3) indicating their survival in both the temperatures.

The results obtained from RNA-seq may not be a good representative of the whole exome since there is a risk of losing genes with low expression. In the present study we have only focused on skin tissue, other tissues might have different expression patterns in response to different temperatures as in the catfish study the liver and gill tissue exposed to heat stress had different results [15]. However, this present study provides a broad understanding of heat response in lumpfish in the varied temperature and to provide early insights into the important processes and pathways that may be helpful for the further studies.

Conclusion

We identified several proteins and genes involved in temperature acclimation in lumpfish. The low level of difference in expression of genes/proteins and no separation between the warm and cold temperature samples indicates that lumpfish can sustain well in both the temperatures used in this study. In addition, this study generated protein and gene sequences that could be used for further studies.

References

1. Altringham JD, Block BA. Why do tuna maintain elevated slow muscle temperatures? Power output of muscle isolated from endothermic and ectothermic fish. J Exp Biol. 1997; 200(20):2617.

2. Johnston IA, Dunn JJ. Temperature acclimation and metabolism in ectotherms with particular reference to teleost fish. Sym Soc Exp Biol. 1987; 41:67-93.

3. Beitinger TL, Bennett WA, McCauley RW. Temperature Tolerances of North American Freshwater Fishes Exposed to Dynamic Changes in Temperature. Environ Biol Fish. 2000; 58(3):237-75.

4. Das T, Pal AK, Chakraborty SK, Manush SM, Chatterjee N, Mukherjee SC. Thermal tolerance and oxygen consumption of Indian Major Carps acclimated to four temperatures. J Therm Biol. 2004; 29(3):157-63.

5. Dülger N, Kumlu M, Türkmen S, Ölçülü A, Tufan Eroldoğan O, Asuman Yılmaz H, Öçal N. Thermal tolerance of European Sea Bass (Dicentrarchus labrax) juveniles acclimated to three temperature levels. J Therm Biol. 2012; 37(1):79-82.

6. Cross EE, Rawding RS. Acute thermal tolerance in the round goby, Apollonia melanostoma (Neogobius melanostomus). J Therm Biol. 2009; 34(2):85-92.

7. Deane EE, Woo NYS. Cloning and characterization of the hsp70 multigene family from silver sea bream: Modulated gene expression between warm and cold temperature acclimation. Biochem Bioph Res Co. 2005; 330(3):776-83.

8. Podrabsky JE, Somero GN. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish <em>Austrofundulus limnaeus</em&gt. J Exp Biol. 2004; 207(13):2237.

9. Logan CA, Somero GN. Effects of thermal acclimation on transcriptional responses to acute heat stress in the eurythermal fish Gillichthys mirabilis (Cooper). AM J PHYSIOL-REG I. 2011; 300(6):R1373-R83.

10. Pérez-Casanova JC, Rise ML, Dixon B, Afonso LOB, Hall JR, Johnson SC, Gamperl AK. The immune and stress responses of Atlantic cod to long-term increases in water temperature. Fish Shellfish Immunol. 2008; 24(5):600-09.

11. Imsland AK, Reynolds P, Eliassen G, Hangstad TA, Nytrø AV, Foss A, Vikingstad E, Elvegård TA. Feeding preferences of lumpfish (Cyclopterus lumpus L.) maintained in open net-pens with Atlantic salmon (Salmo salar L.). Aquaculture. 2015; 436(Supplement C):47-51.

12. Nytrø AV, Vikingstad E, Foss A, Hangstad TA, Reynolds P, Eliassen G, Elvegård TA, Falk-Petersen I-B, Imsland AK. The effect of temperature and fish size on growth of juvenile lumpfish (Cyclopterus lumpus L.). Aquaculture. 2014; 434(Supplement C):296-302.

13. Patel DM, Brinchmann MF. Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). Biochem Biophys Rep. 2017; 9:217-25.

14. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011; 12(1):323.

15. Liu S, Wang X, Sun F, Zhang J, Feng J, Liu H *et al*. RNA-Seq reveals expression signatures of genes involved in oxygen transport, protein synthesis, folding, and degradation in response to heat stress in catfish. Physiol Genom. 2013; 45(12):462-76.

16. Perez-Sanchez J, Bermejo-Nogales A, Calduch-Giner JA, Kaushik S, Sitja-Bobadilla A. Molecular characterization and expression analysis of six peroxiredoxin paralogous genes in gilthead sea bream (*Sparus aurata*): insights from fish exposed to dietary, pathogen and confinement stressors. Fish Shellfish Immunol. 2011; 31(2):294-302.

17. Tacchi L, Secombes CJ, Bickerdike R, Adler MA, Venegas C, Takle H, Martin SAM. Transcriptomic and physiological responses to fishmeal substitution with plant proteins in formulated feed in farmed Atlantic salmon (Salmo salar). BMC Genomics. 2012; 13(1):363.

18. Johnson ES. Protein Modification by SUMO. Ann Rev Biochem. 2004; 73(1):355-82.

19. Yoo CY, Miura K, Jin JB, Lee J, Park HC, Salt DE, Yun D-J, Bressan RA, Hasegawa PM. SIZ1 Small Ubiquitin-Like Modifier E3 Ligase Facilitates Basal Thermotolerance in Arabidopsis Independent of Salicylic Acid. Plant Physiol. 2006; 142(4):1548-58.

20. Rajan B, Fernandes JMO, Caipang CMA, Kiron V, Rombout JHWM, Brinchmann MF. Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. Fish Shellfish Immunol. 2011; 31(2):224-31.

21. Rajan B, Lokesh J, Kiron V, Brinchmann MF. Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*. BMC Vet Res. 2013; 9(1):103.

22. Lu C-Y, Hsu C-Y. Ambient temperature reduction extends lifespan via activating cellular degradation activity in an annual fish (Nothobranchius rachovii). Age. 2015; 37(2):33.

23. Young JC, Agashe VR, Siegers K, Hartl FU. Pathways of chaperone-mediated protein folding in the cytosol. Nat Rev Mol Cell Biol. 2004; 5:781.

24. Vainberg IE, Lewis SA, Rommelaere H, Ampe C, Vandekerckhove J, Klein HL, Cowan NJ. Prefoldin, a Chaperone that Delivers Unfolded Proteins to Cytosolic Chaperonin. Cell. 1998; 93(5):863-73.

25. Jang DG, Sim HJ, Song EK, Medina-Ruiz S, Seo JK, Park TJ. A thioredoxin fold protein Sh3bgr regulates Enah and is necessary for proper sarcomere formation. Dev biol. 2015; 405(1):1-9.

26. Huth TJ, Place SP. Transcriptome wide analyses reveal a sustained cellular stress response in the gill tissue of Trematomus bernacchii after acclimation to multiple stressors. BMC Genomics. 2016; 17(1):127.

27. Kammer AR, Orczewska JI, Brien KM. Oxidative stress is transient and tissue specific during cold acclimation of threespine stickleback. J Exp Biol. 2011; 214(8):1248.

28. Lushchak VI. Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol. 2011; 101(1):13-30.

29. Le Hir H, Gatfield D, Braun IC, Forler D, Izaurralde E. The protein Mago provides a link between splicing and mRNA localization. EMBO Rep. 2001; 2(12):1119-24.

30. Gracey AY, Troll JV, Somero GN. Hypoxia-induced gene expression profiling in the euryoxic fish Gillichthys mirabilis. P Natl Acad Sci USA. 2001; 98(4):1993-98.

31. Montfort J, Le Cam A, Gabillard J-C, Rescan P-Y. Gene expression profiling of trout regenerating muscle reveals common transcriptional signatures with hyperplastic growth zones of the post-embryonic myotome. BMC Genomics. 2016; 17:810.

32. Hussainzada N, Lewis JA, Baer CE, Ippolito DL, Jackson DA, Stallings JD. Whole adult organism transcriptional profiling of acute metal exposures in male Zebrafish. BMC Pharmacol Toxicol. 2014; 15(1):15.

33. Bagatini MD, Martins CC, Battisti V, Gasparetto D, da Rosa CS, Spanevello RM *et al*. Oxidative stress versus antioxidant defenses in patients with acute myocardial infarction. Heart Vessels. 2011; 26(1):55-63.

34. Ghanbari R, Zarei M, Ebrahimpour A, Abdul-Hamid A, Ismail A, Saari N. Angiotensin-I Converting Enzyme (ACE) Inhibitory and Anti-Oxidant Activities of Sea Cucumber (Actinopyga lecanora) Hydrolysates. Int J Mol Sci. 2015; 16(12):28870-85.

35. Krasnov A, Skugor S, Todorcevic M, Glover KA, Nilsen F. Gene expression in Atlantic salmon skin in response to infection with the parasitic copepod Lepeophtheirus salmonis, cortisol implant, and their combination. BMC Genomics. 2012; 13(1):130.

36. Quinn NL, McGowan CR, Cooper GA, Koop BF, Davidson WS. Ribosomal genes and heat shock proteins as putative markers for chronic, sublethal heat stress in Arctic charr: applications for aquaculture and wild fish. Physiol Genom. 2011; 43(18):1056-64.

37. Pytharopoulou S, Sazakli E, Grintzalis K, Georgiou CD, Leotsinidis M, Kalpaxis DL. Translational responses of Mytilus galloprovincialis to environmental pollution: Integrating the responses to oxidative stress and other biomarker responses into a general stress index. Aquat Toxicol. 2008; 89(1):18-27.

38. Schneider WJ, Nimpf J, Bujo H. Novel members of the low density lipoprotein receptor superfamily and their potential roles in lipid metabolism. Curr Opin Lipidol. 1997; 8(5).

39. St Clair RW, Beisiegel U. What do all the apolipoprotein E receptors do? Curr Opin Lipidol. 1997; 8(5).

40. Hu J, You F, Wang Q, Weng S, Liu H, Wang L, Zhang P-J, Tan X. Transcriptional Responses of Olive Flounder (Paralichthys olivaceus) to Low Temperature. PloS One. 2014; 9(10):e108582.

41. Scott GR, Johnston IA. Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. P Natl Acad Sci USA. 2012; 109(35):14247-52.

42. Zhang X, Wen H, Wang H, Ren Y, Zhao J, Li Y. RNA-Seq analysis of salinity stress–responsive transcriptome in the liver of spotted sea bass (Lateolabrax maculatus). PloS One. 2017; 12(3):e0173238.

43. Concha MI, Molina Sa, Oyarzún C, Villanueva J, Amthauer R. Local expression of apolipoprotein A-I gene and a possible role for HDL in primary defence in the carp skin. Fish Shellfish Immunol. 2003; 14(3):259-73.

44. Craig TA, Zhang Y, McNulty MS, Middha S, Ketha H, Singh RJ *et al*. Research Resource: Whole Transcriptome RNA Sequencing Detects Multiple 1α,25-Dihydroxyvitamin D(3)-Sensitive Metabolic Pathways in Developing Zebrafish. Molecular Endocrinology. 2012; 26(9):1630-42.

45. Smith S, Bernatchez L, Beheregaray LB. RNA-seq analysis reveals extensive transcriptional plasticity to temperature stress in a freshwater fish species. BMC Genomics. 2013; 14(1):375.

46. Rajesh S, Kiruthika J, Ponniah AG, Shekhar MS. Identification, cloning and expression analysis of Catechol-O-methyltransferase (COMT) gene from shrimp, Penaeus monodon and its relevance to salinity stress. Fish Shellfish Immunol. 2012; 32(5):693-99.

47. Romero A, Dios S, Poisa-Beiro L, Costa MM, Posada D, Figueras A, Novoa B. Individual sequence variability and functional activities of fibrinogen-related proteins (FREPs) in the Mediterranean mussel (Mytilus galloprovincialis) suggest ancient and complex immune recognition models in invertebrates. Dev Comp Immunol. 2011; 35(3):334-44.

48. Moss SE, Morgan RO. The annexins. Genom Biol. 2004; 5(4):219-19.

49. Lizarbe MA, Barrasa JI, Olmo N, Gavilanes F, Turnay J. Annexin-Phospholipid Interactions. Functional Implications. Int J Mol Sci. 2013; 14(2):2652-83.

559 50. Ozerova SG, Minin AA. A study of proteins of annexin group in early fish
560 development. IV. Identification of calcium-binding proteins in zebrafish egg by
561 mass spectrometry. Russ J Dev Biol. 2008; 39(3):181.

562 51. Perretti M, Flower RJ. Annexin 1 and the biology of the neutrophil. J Leukocyte
563 Biol. 2004; 76(1):25-29.

564 52. Apte SS. A Disintegrin-like and Metalloprotease (Reprolysin-type) with
565 Thrombospondin Type 1 Motif (ADAMTS) Superfamily: Functions and
566 Mechanisms. J Biologic Chem. 2009; 284(46):31493-97.

567 53. Choi GCG, Li J, Wang Y, Li L, Zhong L, Ma B *et al*. The Metalloprotease
568 ADAMTS8 Displays Antitumor Properties through Antagonizing EGFR–MEK–
569 ERK Signaling and Is Silenced in Carcinomas by CpG Methylation. Mol Can Res.
570 2014; 12(2):228.

571 54. Buckley BA, Gracey AY, Somero GN. The cellular response to heat stress in the
572 goby Gillichthys mirabilis: a cDNA microarray and protein-level analysis. J Exp
573 Biol. 2006; 209(14):2660.

574 55. Allen PB, Greenfield AT, Svenningsson P, Haspeslagh DC, Greengard P. Phactrs
575 1–4: A family of protein phosphatase 1 and actin regulatory proteins. P Natl Acad
576 Sci USA. 2004; 101(18):7187-92.

577 56. Fils-Aimé N, Dai M, Guo J, El-Mousawi M, Kahramangil B, Neel J-C, Lebrun J-J.
578 MicroRNA-584 and the Protein Phosphatase and Actin Regulator 1 (PHACTR1), a
579 New Signaling Route through Which Transforming Growth Factor-β Mediates the
580 Migration and Actin Dynamics of Breast Cancer Cells. J Biol Chem. 2013;
581 288(17):11807-23.

582 57. Paffenholz R, Franke WW. Identification and localization of a neurally expressed
583 member of the plakoglobin/armadillo multigene family. Differentiation. 1997;
584 61(5):293-304.

585 58. Anastasiadis PZ, Reynolds AB. The p120 catenin family: complex roles in
586 adhesion, signaling and cancer. J Cell Sci. 2000; 113(8):1319.

587 59. Patwari P, Higgins LJ, Chutkow WA, Yoshioka J, Lee RT. The Interaction of
588 Thioredoxin with Txnip: Evidence for Formation of a Mixed Disulfide by Disulfide
589 Exchange. J Biologic Chem. 2006; 281(31):21884-91.

590 60. Falfushynska HI, Phan T, Sokolova IM. Long-Term Acclimation to Different
591 Thermal Regimes Affects Molecular Responses to Heat Stress in a Freshwater Clam
592 Corbicula Fluminea. Sci Rep. 2016; 6:39476.
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Author Contributions

TK conceived the study, TK, MFB and MHI designed the study. DMP sampled the tissues, performed the experiments and analysis. NP performed the proteomic analysis in this paper. DMP and TTB analysed the data. DMP wrote the first draf and all authors reviewed the manuscript.

Additional Information

The authors declare no competing financial interests. Additional experimental data will be provided on request.

Table 1. protein name and parameters obtained from MASCOT search list below for lumpsucker acclimation

No	Protein Name/Organism	Acc. No	Theoretical p*I*/Mr	Total score/ ST	SC (%)	FC	Peptide sequences	Gene ontology terms (retrieved from UniProtKB)
1	Small ubiquitin-related modifier 2/ *Notothenia coriiceps*	XP_010770112.1	5.32/10885	58/55	12	0.18	KVAGQDGSIVQFKI	Protein sumoylation (GO:0016925), regulation of prteosomal ubiquitin dependent catabolic process (GO:0032436)
3	Prefoldin subunit 5/ *Larimichthys crocea*	KKF32533.1	9.30/14604	66/47	13	0.36	KTQLDQEIEFLTSSIGQLKV	Protein folding (GO00006457)
5	SH3 domain-binding glutamic acid-rich-like protein/ *Osmerus mordax*	ACO10145.1	4.78/ 13128	153/48	21	0.42	KVYIASSSGSTSIKK KYCGNYEAFFDARE	SH3 domain binding (GO:0017124)
6	Protein mago nashi homolog/ *Danio rerio*	NP_001017700.1	5.95 /17269	45/21	7	0.45	KIGSLIDVNQSKD	Regulation of translation (GO:0006417), RNA export from nucleus (GO:0006405)
8	Proteasome subunit beta type 1-A/ *Osmerus mordax*	ACO09437.1	7.00/26440	208/49	15	0.66	RDVYTGDALRI RLSEGYSIHSRD KTMTSGAIAAMLSTILYGRR	Proteasome mediated ubiquitin dependent protein catabolic process (GO:0043161)
9	Retinoblastoma binding protein 4, like/ *Danio rerio*	AAI54779.1	4.71/48095	168/48	6	1.51	KGAEFGGFGSVSGKI KTPTSDVLVFDYTKH	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest (GO:0006977)
10	Angiotensinogen-like/ *Neolamprologus brichardi*	XP_006783765.1	5.19/52208	210/48	5	2	KTLQGINSLVDDGPKD KDEITTQVWAFTRQ KTLQGINSLVDDGPK DEITTQVWAFTRQ	Regulation of systemic arterial blood pressure by renin angiotensin (GO:0003081)/ *Scleropagus formosus*

11	Microfibril-associated glycoprotein 4-like/ *Cyprinus carpio*	XP_018936831.1	7.51/ 28240	65/16	4	2.09	KDKGGWTVIQRR	Regulation of collagen metabolic process (GO:0010712)/ *Rattus rattus*
12	Apolipoprotein E/ *Oplegnathus fasciatus*	ACF21982.1	4.93/31040	191/19	20	2.19	RSTEYLGELKT RNTVATYLGELQSRT KIDELTELLSPYATQIRE RTQAEGLGQQLETQAEGLKT	Lipoprotein biosynthetic process (GO:0042158)
14	40S ribosomal protein SA/ *Salmo salar*	NP_001134397.1	4.93/ 35043	118/21	20	2.36	RLLIVTDPRA MSGGLDVLQMKE KSDGVYIINLKK RYVDIAIPCNNKG KFASATGATTFHGRF RFTPGTFTNQIQAAFRE RAIVAIENPADVCVISSRN	rRNA processing (GO:0006364), translation (GO:0006412)
15	Annexin A3/ *Larimichthys crocea*	KKF17944.1	5.19/ 36677	136/49	5	2.58	KTLIEVLTQRS RSEIDLLDIRA	Phagocytosis (GO:0006909), positive regulation to DNA metabolic process (GO:0051054)
16	Catechol O-methyltransferase domain-containing protein 1-like/ *Notothenia coriiceps*	XP_010780849.1	5.45/ 27212	181/19	14	2.76	RTGGIIAIDNVLWSGKV KVVNPAPSDLTSQALDALNKKL KVVNPAPSDLTSQALDALNKK	O-methyltransferase activity (GO0008171)
2, 4, 7, 13,17	Not identified							

ST- significant threshold, SC- sequence coverage, FC- fold change, GO terms are derived from the human orthologues unless otherwise stated the name of the species

Table 2. Trinity assembly statistics

	Stats based on all transcript contigs	Stats based on only longest isoform per gene
Contig N10	7101	5734
Contig N20	5475	3949
Contig N30	4375	2756
Contig N40	3591	1849
Contig N50	2914	1199
Median contig length	575	386
Average contig	1346.15	743.43
Total assembled bases	422303649	158469532

Table 2. Differentially expressed genes in warm temperature (14°C) group compared to cold temperature (8°C)

Contig ID	E-value	Gene name	Organism	Fold change	FDR (padj value)	GO terms (molecular function)
TRINITY_DN25396_c0_g3_i1, **TRINITY_DN25396_c0_g3_i2**	1.023E-40 7.307E-41	Thioredoxin-interacting	*Larmicthys crocea*	-0.659 -0.657	0.02 0.02	Ko04621 (NOD-like receptor signalling pathway), K20960 (thioredoxin pathway)
TRINITY_DN30663_c0_g1_i2, **TRINITY_DN30663_c0_g1_i5**	0E0 0E0	Phosphate and actin regulator	*Oreochromis niloticus*	-0.675 -0.678	0.03 0.03	Cytoskeleton protein binding (GO:0030234)
TRINITY_DN31802_c2_g1_i1, **TRINITY_DN31802_c2_g1_i2,** **TRINITY_DN31802_c2_g1_i3,** **TRINITY_DN31802_c2_g1_i5,** **TRINITY_DN31802_c2_g1_i7,** **TRINITY_DN31802_c2_g1_i8,** **TRINITY_DN31802_c2_g1_i9,** **TRINITY_DN31802_c2_g1_i10**	0E0 0E0 0E0 0E0 0E0 0E0 0E0 0E0	A disintegrin and metalloproteinase with thrombospondin motifs 8	*Fundulus heteroclitus*	-0.717 -0.698 -0.729 -0.707 -0.715 -0.691 -0.706 -0.695	0.008 0.009 0.01 0.008 0.008 0.009 0.008 0.01	Ion binding (GO:0043167), peptidase activity (GO:0008233)
TRINITY_DN31802_c2_g1_i4	1.15E-140	Metallopeptidase with thrombospondin type 1 8	*Aphyosemion striatum*	-0.755	0.008	Metallopeptidase activity (GO:0008237)
TRINITY_DN31992_c1_g1_i2, **TRINITY_DN31992_c1_g1_i6,** **TRINITY_DN31992_c1_g1_i9**	1.25E-158 4.51E-137 2.61E-137	Proline synthetase co-transcribed homolog (Bacterial)	*Nothobranchius rachovii*	0.270 0.267 0.270	0.04 0.03 0.02	Pyridoxal phosphate binding (GO:0030170)
TRINITY_DN34141_c5_g1_i1, **TRINITY_DN34141_c5_g1_i3,** **TRINITY_DN34141_c5_g1_i5,** **TRINITY_DN34141_c5_g1_i6,** **TRINITY_DN34141_c5_g1_i7,** **TRINITY_DN34141_c5_g1_i9,** **TRINITY_DN34141_c5_g1_i10,** **TRINITY_DN34141_c5_g1_i14**	0E0 0E0 0E0 0E0 1.33E-160 0E0 0E0 0E0	Atherin	*Larmicthys crocea*	0.554 0.567 0.554 0.545 0.554 0.567 0.543 0.564	0.009 0.008 0.008 0.01 0.009 0.008 0.01 0.008	NA
TRINITY_DN36703_c4_g1_i5	5.1E-172	Catenin delta 2 (CTND2)	*Poeciliopsis prolifica*	-0.661	0.02	Protein binding (GO:0005515)
TRINITY_DN36703_c4_g1_i7	0E0	Catenin delta 2	*Fundulus heteroclitus*	-0.730	0.008	Protein binding (GO:0005515)
TRINITY_DN19756_c0_g1_i1, **TRINITY_DN19756_c0_g2_i1**	NA* 	No hits	NA*	-0.727 -0.705	0.01 0.01	

*NA- not available

Figure legends

Figure 1. Differentially expressed proteins. Normalized spot intensities of differentially expressed ($p<0.05$) proteins (Y axis) and differentially expressed protein in X-axis. **(a)** Downregulated spots are 1- small ubiquitin-related modifier 2, 3- prefoldin subunit 5, 5- SH3 domain-binding glutamic acid-rich-like protein, 6- protein mago nashi homolog, 8- proteasome subunit beta type 1-A, 2, 4 and 7 were not identified. **(b)** Upregulated spots are 9- retinoblastoma binding protein 4-like, 10- angiotensin-like, 11- microfibril-associated glycoprotein 4-like, 12- apolipoprotein E, 14- 40S ribosomal protein, 15- annexin A3, 16- catechol methyltransferase domain- containing protein 1-like protein, 13 and 17- not identified. The analysis was based on student's t-test and for data that did not show normal distribution, non-parametric Kolmogorov-Smirnov test (Graph pad Prism 7) was used to understand the difference between the cold vs warm acclimation in lumpfish skin, n=6, fold change ± 1.5; The means ± SD were calculated by Excel software (Microsoft) (asterisk in graph denotes the p-value: *$p< 0.05$, **$p<0.01$, ***$p<0.001$)

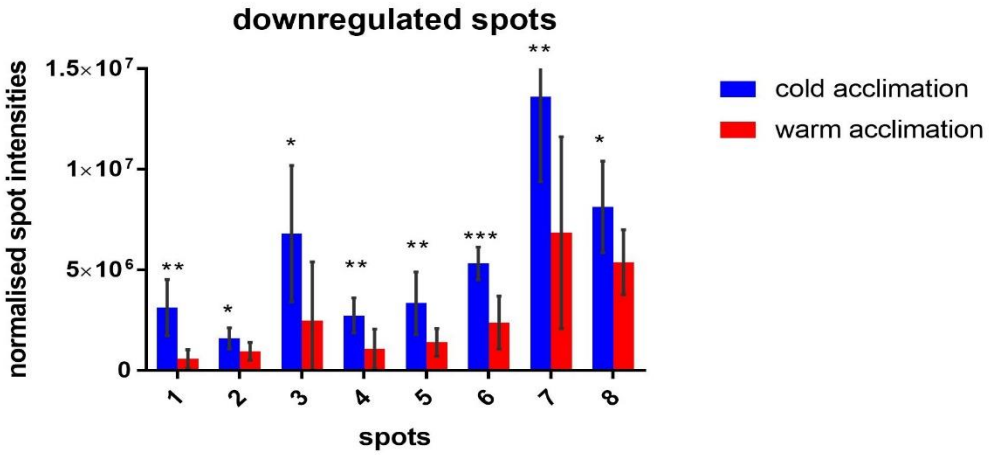
Figure 2. 2D gel image showing the differentially expressed spots. Polyacrylamide gel image (12.5%) for temperature acclimation experiment; spots marked in the gel are differentially expressed spots, spots with yellow circles were identified, spots in blue circles are not identified spots. Molecular weight are marked as kDa. The spot numbers indicates proteins identified as mentioned in Table 1.

Figure 3. Principal component analysis to visualize the sample-to-sample distance. The PCA shows the distance of samples between the two temperature groups. Red dots denote high temperature (14°C) and blue dots denote low temperature (8°C).

Figure 4. Volcano plot showing significantly expressed genes. The red dots indicate the significantly expressed genes in warm temperature group compared to cold temperature group (padj <0.05). The red line denotes that spots above the line have a p value less than 0.05.

Fig 1

a)


downregulated spots
normalised spot intensities
1.5×10^7
1×10^7
5×10^6
0
spots
1
2
3
4
5
6
7
8
cold acclimation
warm acclimation

b)

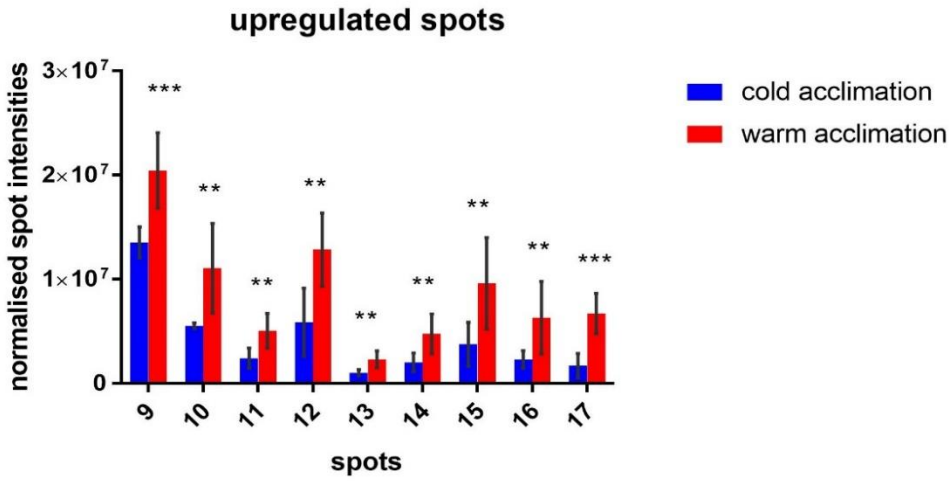

upregulated spots
normalised spot intensities
3×10^7
2×10^7
1×10^7
0
spots
9
10
11
12
13
14
15
16
17
cold acclimation
warm acclimation

Fig 2

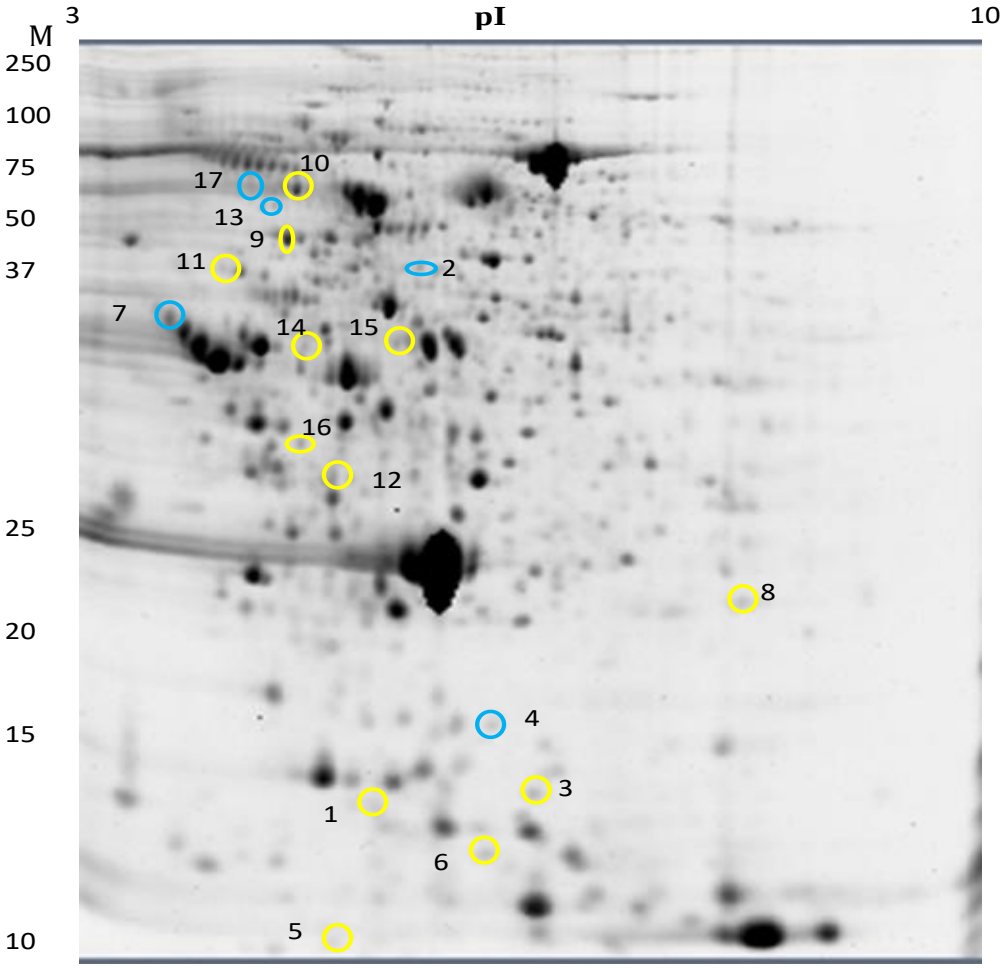

M
3
pI
10
250
100
75
50
37
25
20
15
10
17
10
13
9
11
2
7
14
15
16
12
8
4
3
1
6
5

Fig 3

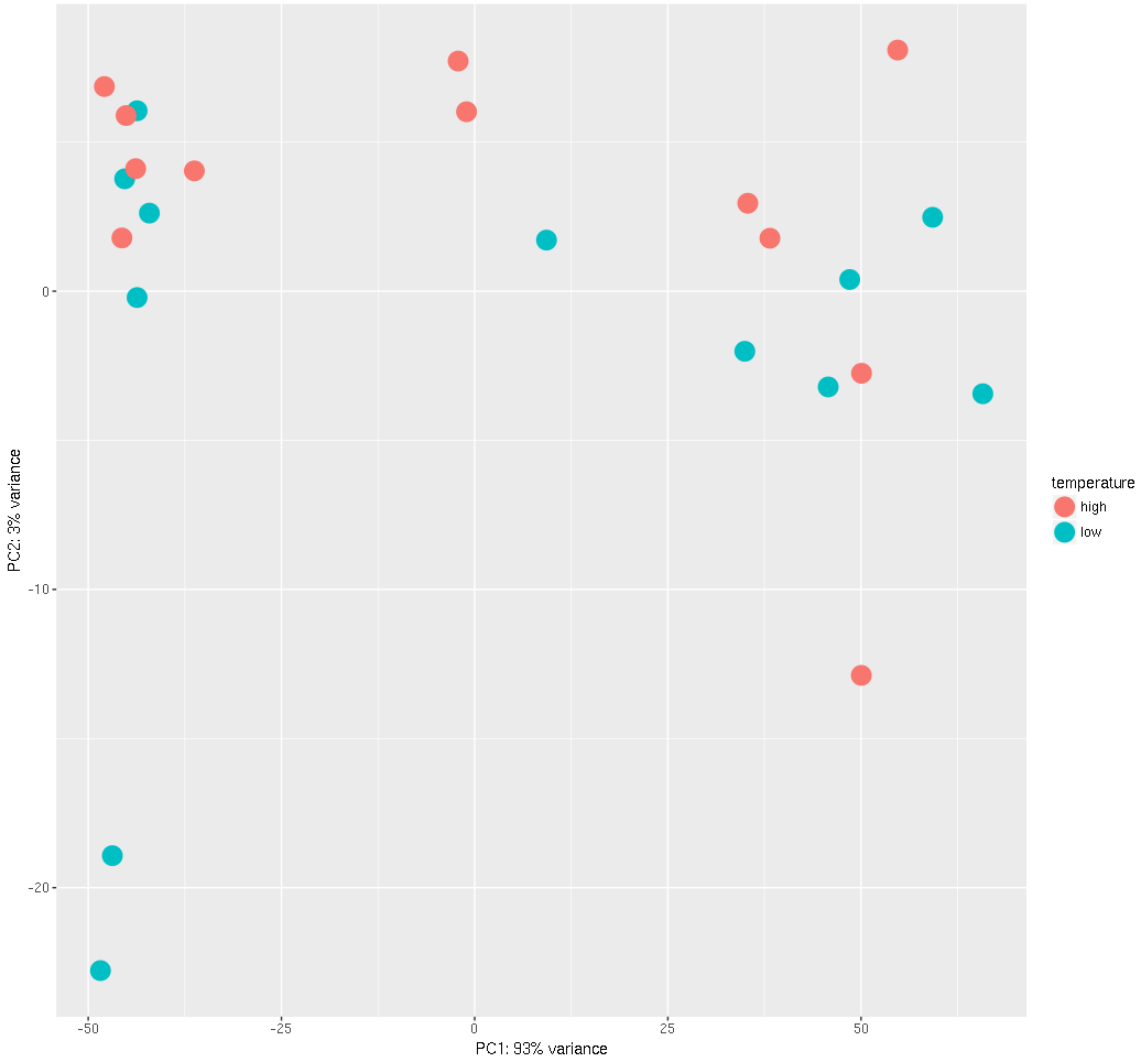

PC2: 3% variance
0
-10
-20
-50
-25
0
25
50
PC1: 93% variance
temperature
high
low

Fig 4