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Stress and immune relevant proteins in lumpsucker (*Cyclopterus lumpus* L.) skin -establishing basic knowledge

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Abbreviations

AGT	Angiotensinogen-like
ANXA3	Annexin A3
APO-E	Apolipoprotein E
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{++}	Calcium
$CaCl_2$	Calcium chloride
CHAPS	(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
cm	Centimeter
COMTD1	Catechol O-methyltransferase domain-containing protein 1-like,
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia (lat.)*, for Example
ESI-Quad-TOF	Electrospray ionisation quadrupole time-of-flight
EST	Expressed sequence tag
FELASA	The Federation of European Laboratory Animal Science Associations
FAO	Food and agricultural organisation
FBA	Faculty of bioscience and Aquaculture
g	Centrifugal force in gravity
h	Hours
IPG	Immobilized pH gradient
l	litre
kDa	Kilodalton
kg	Kilogram
LC-MS/MS	Liquid Chromatography - Tandem Mass Spectrometry
M	Molar
mA	Milliamps
MAGOH	Protein mago nashi homolog
MFAP4	Microfibril-associated glycoprotein 4-like
mM	Millimolar

Mg^{++}	Magnesium
$MgCl_2$	Magnesium chloride
mg	Milligram
MHC	Major histocompatibility complex
mm	Millimetre
min	Minutes
ml	Millilitre
MS-222	Tricaine methane sulfonate
n	Number
NaCl	Sodium chloride
nm	Nanometre
p70s6k	Phosphoprotein 70 ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PFDN-5	Prefoldin subunit 5
pH	Potential of hydrogen
p*I*	Isoelectric point
ppm	Parts per million
PSMB1-A	Proteasome subunit beta type 1-A
RBBP4	Retinoblastoma binding protein 4, like
RNA, mRNA	Ribonucleic acid, messenger RNA
RPS	40S ribosomal protein
s	Seconds
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3BGRL3	SH3 domain-binding glutamic acid-rich-like protein
sp., spp.	Species
SUMO-2	Small ubiquitin-related modifier 2
TBS	Tris buffered saline
TCA	Trichloro acetic acid
Tris-HCL	Trisaminomethane hydrochloride
TSA	Tryptone soya agar
TSB	Tryptic soya broth
w/v	Weight per volume

Zn 2+	Zinc
°C	Degree celsius
%	Percentage
β	Beta
µg	Microgram
µl	Microlitre
Ω	Ohm
µA	Microampere
&	And
2DE-MS	2-dimensional gel electrophoresis followed by mass-spectrometry identification

Abstract

In fish, skin serves as a first line barrier to the microorganism and protect from physical damage. Environmental stress known to impact the skin condition of fish, results in changes in a wide range of physiological parameters, immunology and affect the behaviour. This study describes protein changes in response to temperature acclimation stress in the skin of the lumpsucker by using 2D gel coupled with tandem mass spectrometry. The identified differentially expressed proteins spots were identified by homology searches across the databases SwissProt, NCBIprot and Actinopterygii EST using MASCOT. The identified differentially expressed proteins between the two acclimation temperatures, cold (8 ℃) and warm (14 ℃) and were involved in cellular function: protein folding (PFDN-5); protein degradation (PSMB1-A, SUMO-2), protein biosynthesis (MAGOH, RPS, SH3BGRL3, RBBP4, AGT); energy metabolism (APO-E, COMTD1), cytoskeletal reorganisation (MFAP4) and cell proliferation and apoptosis (ANXA3).

Mannose binding proteins are involved in the pathogen recognition and play roles in innate immunity by neutralization of pathogens through protein-carbohydrate interactions. In this study mannose binding proteins were isolated from lumpsucker skin by using mannose sepharose affinity chromatography followed by sephacryl S 200 HR gel filtration and six bands were present in 1D SDS-PAGE. Among the six mannose binding proteins bands in the 1D gel, five were identified by a homology searches across the MASCOT databases, to be transferrin, glial fibrillary acidic protein, a1-antirypsin, fucose binding lectin and pentraxin fusion like protein. The hemagglutination activity of isolated proteins was Ca^{++} dependent and agglutination was observed with *Aeromonas salmonicida* suggesting mannose binding proteins moieties in the surface of the bacterial cell. Over all, this study gives early insights in the molecular response in lumpsucker after change in temperature to provide basic information into the important process of acclimation this study also shows pathogen recognition of mannose binding proteins, as well as skin extracts. The results contribute knowledge on the skin barrier, important in innate immunity that may be helpful for the further studies.

1. Introduction

Aquaculture industry in Norway is dominated by the finfish aquaculture rather than shellfish and Norway is the leading country in Atlantic salmon production and one of the intensive culture marine finfish. The world per capita fish consumption increased from 14.4 kg to 19.7 kg in 2013 and global total capture fishery production in 2014 was 93.4 million tonnes, of which 81.5 million tonnes from marine waters and 11.9 million tonnes from inland waters (FAO, 2016). The global production of farmed Atlantic salmon (*Salmo salar*) is the fastest growing food production system in the world (Shepherd, 2014). Currently the sealouse (mainly *Lepeophtheirus salmonis*) a marine ectoparasite causing major problems to the Norwegian salmon industry, which believed to have significant negative impact on wild salmon as well other species. Once Atlantic salmon skin exposed to sealouse and are highly susceptible to bacterial and viral infections through suppression of host immune responses and damage to the host skin (Frazer et al., 2012). Salmon industry has been struggling with the issue of sea louse and has relied heavily upon the usage of chemotherapeutics. In the salmon farms sea louse control relies on the use of licensed veterinary drugs (Costello, 2009) and currently, major controlling method by chemical treatments were form an increasing risk of resistance by sea louse (Aaen et al., 2015) and resistance has recently been reported in Chile (Bravo, 2009), Scotland and Canada (Jones et al., 2013) and cause environmental impact as well. To compensate the chemicals usage in farm and cost of controlling sealouse inclined without effective vaccine against sealouse, the biological way of control of sea louse through use of cleaner fish has recently become a feasible alternative way for the increased occurrence of resistant lice to medicine and were a sustainable way of salmon aquaculture.

1.1. Biological characteristic and distribution of lumpsucker a cleaner fish

Lumpsucker (*Cyclopterus lumpus* Linnaeus, 1758) is recently introduced fish to the Norwegian salmon aquaculture. The utilization of lumpsucker as a cleaner fish to expel the salmon lice. These fishes are well suited for cold water and are widely distributed in the boreal region on both sides of the North Atlantic and are found in the Barents Sea, White Sea, along the Norwegian, Danish, Dutch, Belgian, United Kingdom, French, and Spanish coasts and as far south as the northern coast of Portugal (Davenport, 1985, Stevenson, 1988, Nytrø et al., 2014). The lumpfish is a scale-less, short and tough-skinned fish, three rows of bony plates/ridges on both sides to the body. Each of these ridges appeared like a pointed tubercle and it is studded on the skin. One of the row of bony ridges originated over the eye, one close to the pectoral fin and one marking the line of transition between side and belly (Davenport, 1985). Effortlessly recognized through cartilaginous skeleton and subcutaneous jelly tissue covers entirely in dorsal crest, which together makes the density of

the body close to that of seawater and gives buoyancy for the lacking swim bladder in lumpsucker (Davenport and Kjørsvik, 1986). Both male and female varies in colour and size, males normally brighter in appearance and female typically larger in size. Special characteristic of the fish is that during breeding season fish change its body colouration, contains bright pink or red colouration whereas female remains in the same colour (blue green). Males usually smaller and mature earlier, develop a larger suction cup (Hedeholm et al., 2014). The lumpsucker has generally been considered an unpalatable fish for human consumption but heavier female lumpsucker (Fig. 1) are usually fished commercially for their roe, which is used alternative for the caviar production and during present study it was noticed that female contain ripe ovary (light pink roe) which can fill two sides of the body cavity in fish. The ripe females yield between 15 % and 36 % of roe by weight (Mitamura et al., 2007, Hedeholm et al., 2014). During breeding season, late winter and early spring these fishes move from offshore to shallow spawning ground (Mitamura et al., 2012) and after fertilisation eggs are guarded by male until hatching. Lumpsucker is generally sluggish bottom dwelling fish, most of the time attached to the bottom substratum. These fishes are look quite unusual compared to other fishes due to the modification of pelvic fins to constitute a ventral suction disc allowing specimens to rest on vegetation, rocky substrate and algae (Davenport, 1985).

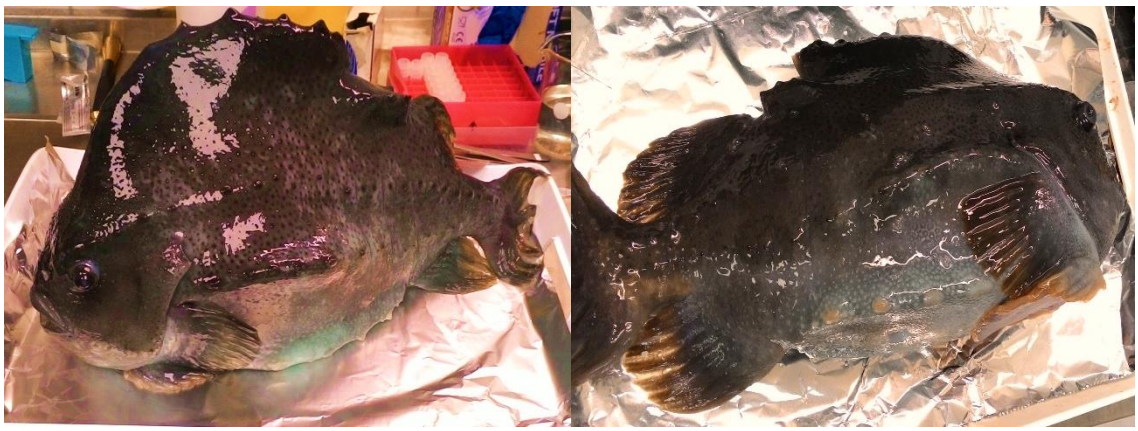


Figure 1. Lumpsucker (*Cyclopterus lumpus*); with average weight of 3.5 to 4 kg used for mucus sampling for hemmaglutination and bacterial agglutination test.

1.1.1. Lumpsucker a cleaner fish with importance in aquaculture

The use of the cleaner fish as biological agents for salmon louse control has dramatically incremented in the Norwegian and Scottish aquaculture. Lumpsucker is one of the newly introduced cleaner fish to delousing the sealice in the salmon farming. Lumpsucker were complementary species to ballan wrasse. Wrasse have been used previously used to delouse the

sealice in floating net pens for almost 30 years in the salmon farms (Bjordal, 1991). However, study showed wrasse failure in feeding of sealice to the winter season and do not feed below 6 °C (Kelly et al., 2014), limitation was in the winter as a cleaner fish to compensate the issues in winter and spring season another cleaner fish has been introduced, that offers the greatest potential (Imsland et al., 2014a, Imsland et al., 2014b, Imsland et al., 2014c, Imsland et al., 2015a, Imsland et al., 2015b). Lumpsucker importance over wrasse by continuing feeding at temperatures as low as 4 °C (Nytrø et al., 2014). Lumpsucker is poorly studied fish and about their behaviour and biology literature is limited. Lumpsucker were ready for deployment in salmon farms in as little as 4 months, much sooner than ballan wrasse which typically require 1.5 years to get into the farm (Powell et al., 2017). Studies revealed that lumpsucker appeared to be particularly suitable for lice control under Norwegian conditions (Imsland et al., 2014c) as they are active state at low water temperature, in contrast to wrasse species. However, new diseases were common whenever introduction of the new species to the culture environment. However, recently many organizations around the world are researching this fish as a biological way of controlling the sealice in the salmon farming system. Lumpsucker in the salmon farming become essential topics of research as the dimension of aquacultural activities inclined to overcome the challenges, mitigate the negative aspect and longterm sustainability (economical, social and ecological).

1.1.2 Diseases in farmed lumpsucker

Diseases are a major problem with the cultivated fish, due to intensification and high densities the fish could become stressed and exposure to pathogen lead to disease. Physiological stress is one of the most important factor modulating the fish immune system. A wide array of diseases has been plaguing the fish farm and thus, pathogen detection and health management aspects have emerged as a top research priority. Recent year reports showed there were some mortalities when lumpsucker was transferred to the sea, during 2015 several cases of acute mortalities were reported during late summer and autumn, mainly following sea transfer (Bornø, 2016). As the introduction of the lumpsucker species to the salmon farming, its health and welfare issues are continuously being discovered. Some diseases were also observed in the lumpsucker, the following bacterial fish pathogens have been associated with disease in lumpsuckers: atypical *Aeromonas salmonicida*, *Vibrio anguillarum*, *Pasteurella* sp., *Vibrio ordalii*, *Moritella viscosa*, *Tenacibaculum* spp. and *Pseudomonas anguilliseptica* (Bornø and Lie, 2015, Gulla et al., 2015, Alarcón et al., 2016, Bornø, 2016). Many of the recent reports reveal that the cleaner fish mortality is a challenge to their sustainable use and (Nilsen et al., 2014) observed 33 % cumulative mortality noted within six months after transfer to the salmon cage. This results in a significant distortion of

cleaner fish in salmon farms, implicating both adverse effect on animal welfare aspects and economic consequences for the farmers.

I.2. Innate mechanism in fish skin

The immune system protects the organisms from the diseases and in teleost, adaptive immunity is less developed than in mammals. The innate system plays an instructive role in the acquired response (Magnadóttir, 2006) and under normal healthy condition of organisms, by defending itself against invading pathogens. The innate immune system is commonly divided in three; the mucosal/epithelial barrier, the humoral parameters and the cellular components (Uribe et al., 2011). The skin is a largest organ in the fish contain largest mucosal tissue which is always exposed to the environment were microbe is in higher ratios. The skin in the fish believed to be vital role in the innate mechanism, especially mucus which supports the skin by avoiding the invasion foreign body. Different species vary in their skin's structural organisation and function. The integument of fish known to be multifunctional organ, and its components may serve important roles in protection, communication, sensory perception, locomotion, respiration, ion regulation (reviewed by (Marshall and Bellamy, 2010)). The epidermis is a non-keratinized, squamous stratified epithelium that varies in thickness from approximately 3 to 20 cell layers (Ferguson, 1989). Cellular barriers such as macrophages which is an important leukocyte, phagocyte and dendritic cells provide a bridge between innate and acquired immunity which have been found in the skin (reviewed by (Jensen, 2015)). In epidermis, epithelial cells maintain homeostasis through the expression of pattern recognition receptors (PRRs), including lectins, NOD-like receptors (NLRs), and toll-like receptors (TLRs). Mucus in the superficial epithelial cells in the epidermis assist in protecting the skin from the pathogen. Epidermis of fishes may have two different types of glandular cells, namely, goblet cells and club cells (Whitear and Mittal, 1982, Magnadottir, 2010). Mucins are higher molecular weight glycoprotein, present in fish skin contains protein domains with their extensive site O-Glycan attachment. Skin mucus is considered the first line of defense against infection through the epidermis (Raj et al., 2011) and especially skin mucus which contains innate immune factors such as proteases, antibacterial agents and other immune molecules (Suzuki et al., 2003, Easy and Ross, 2009). Other immune molecules in fish mucus include C-reactive protein, immunoglobulins, complement, interferon, calmodulin, interferon and lectins (Martínez- Antón et al., 2006, De Veer et al., 2007, Kitani et al., 2007).

1.3. Fish skin mucus components, their function and modulation

Skin mucus is the humoral barrier for the pathogen, which is a tenacious to most particle and preventing their penetration to the epithelial (epidermal) surface as a biological filter for the fish

skin surface. It is a biological boundary between fish and their external aquatic environment consists of a mucus layer composed of bio-chemically diverse secretions from epidermal cells. Mucus secreting in the skin of the fish by three important cells types goblet cells, sacciform cells and club cells (Zaccone et al., 2001). Proteins in the skin reach to the mucus by various routes, such as classical and non-classical way (reviewed in (Brinchmann, 2016)). Mucus is continuously secreted, it varied among species by their exogenous and endogenous factors and which prevent the stable colonisation of the microbes in the skin and (Van der Marel et al., 2010) observed in the carp, the amount of skin mucus and their glycosylation increased after inclined bacterial load. The complete range of immune factors present within the skin mucus is so far poorly understood. Many antimicrobial peptides (a large group of short polypeptides) and their properties have been identified in fish species (Ángeles Esteban, 2012). The antimicrobial peptides (AMP) involved in the host defense is highly expressed in the skin than in gill and gut been noticed and these AMP involved in the host defense (Gomez et al., 2013) and are also known as host defense peptides. Antimicrobial properties have been noticed in different fishes in the epidermal mucus against infectious pathogens (Kuppulakshmi et al., 2008) also expression level in the epidermal mucus component varied following microbial stress in order to support the defence (Patrzykat et al., 2001). Many antimicrobial peptides and its important part in the skin mucus such as alpha-helical amphipathic peptides, cysteine-rich AMP, they have been reviewed by (Ángeles Esteban, 2012). Proteins such as calpain and keratin been observed in different fish species (Rajan et al., 2013b). Proteolytic enzyme present in skin mucus and play a role in lytic action against pathogen and proteases can also activate and increase the production of other innate immune components present in fish mucus such as antibacterial peptides, complements, or immunoglobulins (Esteban and Cerezuela, 2015). Lactoferrin, a transferrin family are non-heme iron-binding protein present in mucus and can inhibit allergic responses and promote skin immunity (Zaccone et al., 2001). Skin mucus also contains ribosomal proteins have an important role in immunity, 40S ribosomal protein was isolated from the skin of the rainbow trout (Fernandes and Smith, 2002).

1.4.1. Lectin

Lectins are important immune mediator in lower vertebrate and invertebrate, it has a capacity to cell agglutination and precipitation of the glycoconjugates. Lectin is considered one of the essential arms in combating pathogen as a potential antimicrobial agent in the skin mucus. Lectins are a diverse group of proteins that bind specifically to various carbohydrates and are present in every organism, suggesting their role in basic biological functions, including regulatory, adhesive, defence against pathogens (Varki et al., 2009). Agglutination and precipitation properties enable

lectins to mediate different biological processes such as cell-cell interactions (Gabor et al., 2004). Function of lectins is recognition and effector factors, complement activating factors, signalling receptors, and as key role in immune regulators (Vasta and Ahmed, 2008). Although, immune response of lectins in the skin mucus of lumpsucker yet to be known. Some of the lectins in the fish may be present in the intracellular compartments mediating processes, such as splicing of RNA to protein folding and trafficking proteins (Vasta et al., 2011) and some lectins can still be released to extracellular compartment may be in cell surface or soluble components in biological fluids.

1.4.2. Classification of the lectin in fish

Lectins have been classified based on their specific carbohydrate which they recognize. Based on structural homology carbohydrate-recognition domain (CRD) animal lectins been grouped (Vasta et al., 2011) and lectins have been categorised into two major classes, C-type and S-type (Drickamer and Taylor, 1993). S-type is predominantly localised in extracellular (cytoplasm) and specially recognized by β galactosides and are designated as thiol dependent (Kilpatrick, 2002). The C-type superfamily includes the C-type lectins (CTL) and proteins containing C-type lectin-like domain, requires calcium to bind carbohydrate. C-type domains are normally found in animal lectins from serum, extracellular matrix (cell membrane), and membranes Several lectin families been identified in the fish as follows below table.

Table 1. Main categories of lectin identified in fish and their function

Categories	Main features	Function/biological activity	Identified lectins in fishes
C-type lectins Subgroups: collectins, proteoglycan, selectins, endocytic receptors, and the mannose binding lectin (Vasta and Ahmed, 2008)	Ca^{2+} dependent; conserved Ca^{2+} binding site	Innate immunity (collectins); promote phagocytosis, complement activation (MBL); Cell adhesion (selectins) (Kerrigan and Brown, 2009)	Rainbow trout, Catfish, carp, eel Fugu and zebrafish (Singha et al., 2008, Zhang et al., 2000).

S-type lectins (Galectins) **Subgroups**: proto, chimera, and tandem-repeat (Vasta and Ahmed, 2008)	Binding β-galactosides; Ca^{2+} independent activity	Inflammatory response, mediate cell–cell or cell–intercellular matrix interactions in developmental processes	Electric eel, Japanese eel, zebrafish, elasmobranch and teleost fish (Ahmed et al., 2004), European seabass (Poisa-Beiro et al., 2009), Atlantic cod (Rajan et al., 2013a)
F-type lectins F-type motif, either tandemly arrayed or in mosaic combinations with other domains	Affinity for L-fucose, Ca^{2+} independent, non glycosylated	Molecular recognition in innate immunity (Salerno et al., 2009)	European eel, striped bass (Bianchet et al., 2010)
Rhamnose-binding lectins	Bind L-rhamnose and sugars possessing hydroxyls in the same configuration at C_2 and C_4, such as L-arabinose and D-galactose	Antibacterial and nonself-recognition molecules in the innate immune response (Watanabe et al., 2008, Watanabe et al., 2009)	Steelhead trout (Tateno et al., 2002), Spanish mackerel (Terada et al., 2007)
Pentraxins	Ca^{2+} dependent exist in serum as acute phase protein, L-type lectin fold	Response to stress, injury or infection and modulating immune responses (Cook et al., 2005)	Carp, snapper, wolfish, cod, halibut, rainbow trout (Gerwick et al., 2007, Cook et al., 2005)

Calnexin and calreticulin	high-affinity Ca^{2+} binding site and bind Zn2+, binds mannose	Critical roles in MHC class I-related immune function	Rainbow trout, cyprinids (Kales et al., 2007, Bielek, 2008)

Lectins are also classified depending on the binding of the carbohydrate ligand, lectins-carbohydrate interaction can lead to pathogen recognition (mannose binding lectins), immune trafficking (selectins), and immune cell activation (C-type galectins). Some of the important lectin identified in the skin mucus of the fishes are, congerin produced by club cells in skin mucus of the conger eel (*Conger myriaster*) and AJL-1 from the Japanese eel (*Anguilla japonica*) were classified as galectin (Kamiya et al., 1988).

1.4.3. Mannose binding lectins

Mannose binding lectins (MBL) belongs to C-type lectin family and play an important role in complement system activation. Mannose-binding lectins have been shown to bind the 3- and 4-hydroxyl groups of appropriate sugars including N-acetyl-D-glucosamine (GlcNAc), mannose, N-acetyl-mannosamine (ManNAc), fucose and glucose (Turner, 2003). Binding, later activates the complement pathway and pattern recognition receptors (PRRs) which trigger innate immunity through recognizing and binding of pathogen-associated molecular patterns. The complement system in the fish is a crucial component in innate immunity (Boshra et al., 2006). The C-type lectin like receptor (CTLR) also plays important role in the PRRs, CTLR are transmembrane receptors containing one or more C-type lectin domains in the extracellular membrane. CTLR, binds sugar in a calcium dependent manner or independent manner. Mannose recognition lectins have been reported in trout, salmon, carp, rohu, channel catfish, blue catfish and sea lamprey species were reviewed by (Coriolano et al., 2012). Mannose-binding lectin proteins have been found to bind and agglutinate various microorganisms including Gram-positive and Gram-negative bacteria, mycobacterium, viruses and fungi (Tsutsumi et al., 2005). MBL purified from several fishes; rohu (*Labeo rohita*), rainbow trout (*Oncorhynchus mykiss*), sea lamprey (*Petromyzon marinus*), common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), fugu (*Takifugu rubripes*), and turbot (*Scophthalmus maximus*) reviewed in (Zhang et al., 2012). Recently, novel lectin has been identified in the fish are Fucose-type lectin (FTL) which are C-type lectin due to CTLR can be categorized based on conserved amino acid motifs that determine the preference of glycan recognition and Ca^{++} ion coordination. The CTLR that possess an EPN

(Glu-Pro-Asn) amino acid motif in the carbohydrate recognition domain (CRD) prefer to bind and uptake of glycans with equatorial 3- and 4-hydroxyl (OH) groups like mannose, fucose, and/or N-acetylglucosamine (GlcNac) residues, whereas CTLR that express a QPD (Gln-Pro-Asp) motif possess specificity for vertical 4-OH groups like galactose and N-acetylgalactosamine (GalNAc) terminated glycans (Kawauchi et al., 2012, Lee et al., 2011). The FTL constituted large number of proteins exhibiting greater multiples of the F-type motif, either tandemly arrayed or in mosaic combinations with other domains, including a putative transmembrane receptor and form a cylindrical trimer divided into two globular halves: one containing N-terminal CRDs (N-CRDs) and the other containing C-terminal CRDs (C-CRDs) (Bianchet et al., 2010) and form a cross link between sugar structures in microbial pathogens and glycans on the surface of phagocytic cells from the host.

1.5. Temperature stress and immunity in fish

Proper fish health management is important to control and limit diseases. This need the proper understanding of immunology of the fish, specification of pathogen and environment, if there are any alteration in these parameters fish tend to lose its homeostasis. Environmental stress results in changes in a wide range of physiological parameters, immunology and behaviour at the gene and protein level. Lumpsucker is new to aquaculture and the literature background is limited, temperature is one of the significant factors relevant since farmed fish species cannot escape even from the adverse environmental conditions. In the Norwegian salmon farm, temperature influences skin related problems, for example, in cold temperature winter ulcer disease and at high temperature columnaris disease (Jensen, 2015). Species that are living in low temperature thermally stable environments (e.g. high polar regions and deep sea living species) are believed to be less capable to cope with temperature increase (Huey and Hertz, 1984). Skin mucus is exposed to the outer environment and the proteins in the skin mucus are required to maintain their condition during stressor such as change in the temperature and hydraulic pressure (Ogawa et al., 2002). Salmon pens are exposed to warm temperature during summer in the south of Norway coast (Hevrøy et al., 2012) and other regions including Chile, Ireland, Tasmania and the USA. In the pens the fish cannot freely move down to deeper waters or away from the shore to find colder water, hence temperature changes will possibly have effect on the fishes. Fishes are poikilothermic and the water's temperature changes the fish's internal temperature, changes to close to its thermal limit can stress the fish and induce alteration in the immune system. One result is the release of innate immune factors into the serum to compensate the effects of the stressor. During stress, induction of acute phase proteins were observed (Bayne and Gerwick, 2001, Sung et al., 2009).

The stress response develops depend on the intensity of the stressor and its duration, in this way, the stress responses that can suppress or enhance certain pathways of the immune response. There may be chances to delayed or deduced defence mechanism, thus transiently compromising immune defence and resistance to pathogens. During stress condition, studies also found that most of the of fish produce copious amounts of skin and gill mucus (Vatsos et al., 2010). Epidermal layers itself are able to react with the stressor and resulting in the cellular hyperplasia (Uribe et al., 2011). The limit of immunological permissible temperature depends on the species. Skin mucus proteome mapping has been performed in several species such as Atlantic cod (*Gadus morhua*) (Rajan et al., 2011, Rajan et al., 2013b) European seabass (*Dicentrarchus labrax*) and Gilthead seabream (*Sparus aurata*) (Cordero et al., 2015, Jurado et al., 2015, Cordero et al., 2016) and recently for lumpsucker (Patel and Brinchmann, 2017), transcriptomic studies has been investigated the effects of short-term changes in temperature in various fish species (Kassahn et al., 2007). In the salmon both skin and skin mucus were studied for environmental impacts: water temperature change (4 °C, 10 °C and 16 °C), sea lice infection and wound infection (Jensen, 2015). Fishes like Mudsuckers (*Gillichthys mirabilis*) were acclimated for 2 months at 10 ℃ or 25 ℃ to a different salinities, and protein spots over-expressed or under-expressed in gill epithelium of animals acclimated to high temperature reviewed by (Silvestre et al., 2012). In most of the temperature acclimation studies, molecular chaperons are highly expressed and showed a high degree of evolutionary variation of HSP (Heat shock protein) isoforms (Tomanek and Zuzow, 2010). Changes that occur during acclimation are cell cycle arrest, apoptosis, proteolytic protein degradation, acute inflammatory response and catabolism observed in the stress fish reviewed by (Olsvik et al., 2013). Thus, in the present study, we studied of epidermal skin proteins in the acclimatised fishes to understand the effects at the molecular level after temperature stress.

1.6. Methodological aspects

1.6.1. Purification and characterisation of the lectins

Isolation and characterisation of lectins is important for the study of their basic properties and biological function. Animal lectins are of great significance in the field of glycobiology, decoding the glycocode and contributing to the development of various areas of basic and applied bioscience (Kumar and Mittal, 2011, Sharon, 2008). Presently, the number of studies investigating the role of fish lectins in plasma, mucus, gill, gut and eggs using both immunological and molecular biology techniques (Magnadóttir et al., 2010, Bah et al., 2011). For purification of the lectins, affinity chromatography has been widely used (Santos et al., 2009, Leite et al., 2011). In the present study,

mannose sepharose was used to segregate the mannose binding proteins in the skin of the lumpsucker.

1.6.2. Purification of lectins by carbohydrate affinity chromatography

This method varies from classical chromatography methods by using different ligand and matrices. In this technique, carbohydrate absorbent, is used to purify the lectins or glycan-binding proteins. Lectins are bound to carbohydrates ligands noncovalently, reversibly and involving various interactions between absorbent and lectin such as hydrogen bonds, hydrophobic, electrostatic and van der Waals interactions and dipole attraction. The ligands are usually bound relatively weakly, so that the lectin is readily released from an affinity column by competitive elution under proper pH to elute the lectin. Lectins are water soluble, they are therefore extracted from biological samples by proper aqueous buffer with protease inhibitor at 4 °C to avoid degradation during the process. In the present work gravity flow chromatography or fast protein liquid chromatography (FPLC) were performed, where skin with proteins was applied to the carbohydrate affinity absorbent, incubated using proper binding buffer and then eluted using elution buffer.

1.6.3. Absorbent matrices and ligands

In affinity chromatography, a wide range of adsorbents have been used by several studies for lectin purification. In carbohydrate affinity purification, polysaccharide can be used in two ways, adsorbents binding lectins, or as carriers to which ligands can be attached. Both carrier matrix and absorbent matrices have been used in fish lectin purification studies. Among absorbent matices, sepharose and sephadex have been widely used. Recent reports showed that sepharose has been used extensively for isolation of lectins specific for galactosides and related carbohydrates (Cao et al., 2010, Kato et al., 2011, Pohleven et al., 2011).

Table 2. Matrices and ligands used to isolate fish lectins

Ligands and matrix	Species	Biological source, Lectin identified	References
Mannan-agarose	Milk fish (*Chanos chanos),* African catfish *(Clarias gariepinus)*	Serum, mannose binding protein	(Argayosa et al., 2014), (Argayosa et al., 2011)
	Pufferfish	Skin and intestine,	

Mannose-epoxy-activated sepharose 6B	(*Fugu rubripes*)	mannose specific lectin (pufflectin)	(Tsutsui et al., 2005)
Lactose–agarose	Pony fish *(Leiognathus nuchalis)*	Skin mucus, L-rhamnose-binding lectin	(Okamoto et al., 2005)
Epoxyactivated Sepharose 6B	Skate (*Raja kenojei*)	Skin mucus, lactose specific lectin	(Tsutsui et al., 2009)
DEAE-Sepharose FF	Bighead carp (*Aristichthys nobilis*)	Gill, fucose binding lectin	(Pan et al., 2010)
L-fucose–agarose	Gilt head bream (*Sparus aurata*)	Serum, fucose binding lectin	(Cammarata et al., 2007)
Sepharose CL6B, L-fucose–agarose	Sea bass (*Dicentrarchus labrax*)	Serum, F-type lectin	(Salerno et al., 2009)

1.6.4. Proteomics aspects in the study

The field of proteomic's has brought significant advantages to the study of protein and "omic" technologies (genomics, transcriptomics, proteomics, metabolomics) have been widely implemented in the field of aquaculture. Proteomic's is the study related to the protein and peptides, a diverse group of molecules compared with nucleic acids. Proteomic's relies on the electrophoretic or chromatographic techniques to separate the proteins. Proteomics is conducted in the research work for many different purposes such as to understand post-transcriptional and post-translational regulation, and therefore contribute data about an organism's physiological state, which could have been missed by the studies of transcriptome (Rodrigues et al., 2012). When the fish has been exposed to pathogen or stress, triggering a series of changes in the skin mucus visible at proteomic level (Rajan et al., 2013a, Guardiola et al., 2016). The comparison of the protein expression profile for the stressed fishes facilitates identification of complicated changes in the proteome. Comparative proteomics is a field to understand the complexity of biological response to environmental stresses and also to reveal our understanding of the proteins related to various tolerances (Irar et al., 2010). The omics methods could be more powerful tools for investigating

the mode of action of environmental stressors than classical approaches, including physiological, behavioural, or biochemical methods (Lemos et al., 2010, Tomanek, 2005). The mucosal proteome of skin in several fish species was analysed by using the 2DE/MS coupled approach (Ræder et al., 2007, Easy and Ross, 2009, Rajan et al., 2011). Proteins and peptides are highly studied molecules and well established electrophoretic methods, include agarose and polyacrylamide gel electrophoresis, two-dimensional gel electrophoresis (2DE), capillary electrophoresis, isotachophoresis and others (Pergande and Cologna, 2017). In this study, both 1DE and 2DE electrophoresis have been used to identify the molecular mass of the purified proteins and changes in the proteome after treatment. One dimensional electrophoresis (1DE) is most widely used method for purified protein described by (Laemmli, 1970) and in the present study glycine based electrophoresis was used for the proteins purified by affinity column. Gels prepared by polymerisation of the acrylamide by action of cross linking agents, these cross linkage important for segregation of the proteins (Rath et al., 2013). The protein mixtures were separated according to their molecular weight. Before running on the acrylamide gel, proteins should be denatured using reducing agents and the detergent SDS so that proteins will move with respect to their molecular size, higher molecular size at the top and lower at bottom of the gel.

Another method widely used is to separate proteins by their isoelectric point and molecular weight i.e. 2DE and currently, still by far most widely using method in biochemical laboratories and molecular biology to get separation, detection and quantifying large number of protein spots. In this thesis work, 2DE is performed for the protein profile identification for temperature stressed fish. It is one of the leading technique in proteomics, here protein first undergo isoelectric point focusing (IEF) by using IPG strips and separated based on their net charge to different p*I*, later they are separated with respect to their molecular weight in the gel. Visualised proteins spots (samples) gives many possibilities to control the quality of the sample and the reproducibility of the sample preparation process. Uncontrolled protein degradation and solubilisation problems can be easily detected by gel pattern (Rogowska-Wrzesinska et al., 2013). Some bias is observed in 2DE such as difficulty in separating hydrophobic and extreme proteins (Hochstrasser and Sanchez, 2000). Both 1DE and 2DE are stained to visualise the proteins in the acrylamide by using protein stain such as Coomassie, silver staining, Sypro® Ruby and other convenient staining methods. The gel is stained, photographed, scanned or dried on a transparent backing for a record of the position and intensity of each band (Steinberg, 2009) or spot. Later detected spots in 2DE and bands in 1DE are analysed by mass spectrometry method, it is fast, sensitive and reproducible method and when coupled with bioinformatics can be used to identify proteins. The spots

visualised in 2DE after stain were further analysed by powerful and user-friendly data analysis by means of computer algorithms. Several software packages have been developed such as Melanie, PDQuest, Z3, and Progenesis to facilitate rapid, accurate and objective analysis of 2DE gels reviewed by (Rosengren et al., 2003). In the study, PDQuest (Bio-Rad, USA) is used to check differential expression of proteins. Proteome analysis can be performed in two different strategies, called bottom-up and top-down approaches (Yates et al., 2009). In bottom-up approach, analysed significant protein spots are proteolyzed. The digest mixture from a spot provides a set of peptide molecular masses for a Peptide Mass Fingerprint to search in mass spectrometric analysis (MS). In the top-down, proteins are characterized by MS without proteolysis. Identified ions in mass spectrometer (MS/MS) is then analysed by using MASCOT database (NCBIprot and SWISSprot) and peptide sequences are identified, then the set of peptide sequences is used to infer which proteins may have been present in the sample.

2. Objective of the study

The skin is the largest organ in the fish and important for homeostasis. Immune relevant proteins are found in the skin mucosa and lectins are sugar binding molecules found here with important functions in pathogen recognition, complement activation, agglutination and hemagglutination. In previous work at FBA, differentially expressed proteins have been identified in pathogen stressed Atlantic cod and lectin were isolated from Atlantic cod and Atlantic salmon skin mucus (Rajan et al., 2011, Rajan et al., 2013b) and (Patel, 2014). In a recent study of the skin mucus proteome of lumpsucker a mannose binding lectin was identified, but not isolated and characterised (Patel and Brinchmann, 2017). The main aim of the present study was to identify the temperature stressed lumpsucker skin proteins and isolate and characterise mannose binding proteins from the skin.

Specific objectives in the study were as follows,

- Identification of potential upregulated or downregulated proteins in the skin of temperature acclimated fish by using proteomics, mass spectrometry and bioinformatics tools
- Isolation of mannose binding proteins from the lumpsucker skin
- Identification and characterisation of the isolated mannose binding proteins using mass spectrometry and bioinformatics tool
- Functional characterisation of identified mannose binding proteins to check for hemagglutination and bacterial agglutination

3. Materials and methods

3.1. Chemicals used in the study:

Chemicals used in the study were as follows: MS-222 (Finquel vet. 100%, ScanVacc); PBS (Sigma-Aldrich); protease inhibitor (GE Healthcare, USA); TCA (Sigma-Aldrich); DTT (VWR, Life science); Urea (Merck, Norway); CHAPS (AMRESCO, life science)); BioLyte (Bio-Rad); Bromophenol blue (Sigma-Aldrich); Tris-HCl (pH 8.8 (Bio-Rad)); Glycerol (Alfa Aesar-ThermoFisher Scientific)); Iodoacetamide (Bio-Rad); Methanol (Merck, Norway); Acetic acid (Merck, Norway); Sypro® Ruby Protein gel stain (Life Technologies, USA); NaCl (Merck, Norway); $CaCl_2$ (Sigma-Aldrich); $MgCl_2$ (Sigma-Aldrich); Mannose sepharose resins (GALAB Technologies, Germany); Laemmli buffer (Bio-Rad); β-mercaptoethanol (Bio-Rad); Precision Plus Protein™ Kaleidoscope™ (Bio-Rad)); Colloidal Coomassie (G-250 (Bio-Rad)); trypsin EDTA (Sigma-Aldrich); EDTA (Sigma-Aldrich); Glutaraldehyde (Sigma-Aldrich); Glycine (Sigma-Aldrich); DAPI (Sigma-Aldrich)); TSA (Sigma-Aldrich); Paraformaldehyde (Merck, Norway); TSB (Sigma-aldrich) and BSA (Sigma-Aldrich).

3.2. Maintenance of fish:

In this study, for the temperature acclimation experiment, hatchery reared lumpsucker was brought to Mørkvedbukta Research Station, (WGS84: 67°16'41.7"N 14°33'26.8"E) Nord University, Norway by the end of May 2014 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 before holding at seawater temperature of 7-8 °C and fed with commercial feed Amber Neptun 3 mm. Throughout the experiment period the oxygen content in the water was maintained at 80 % and the photoperiod was 24:0 (light day).

3.2.1. Cold and warm temperature acclimation of lumpsucker:

The acclimation set-up (Fig. 2a) consisted of four circular acclimation tanks of grey fiber-glass units, each with a volume of 400 l with a water flow of 150 l/hour. A total of 40 lumpsuckers were randomly allocated from holding tanks to the four acclimation tanks. Of these available fish, n = 40 used in this study (n = 20 cold acclimated, n = 20 warm acclimated). During the replicate, 10 fishes were held in each of the four acclimation tanks. Among four tanks, two of the acclimation tanks were held at a seawater temperature of approximately 8 °C, while the other two received additional heated seawater from the main tank to keep the temperature at 14 °C. The temperature in the warm acclimation tanks were gradually increased from the holding temperature (7-8 °C) just after lumpsuckers were transferred to the tank and reached a final temperature of 14 °C during

48 h. The temperature acclimation was continued until day 7 after temperature started to increase. Experimental conditions during the acclimation period are shown in Table 3.

Table 3. Experimental conditions during the acclimation period. Means ± standard deviation of temperatures (°C) and oxygen saturation (%), measured in the tank outlet. Fish (n) is the total number of fish per acclimation tank.

Replicate	Tank	n	Temperature (°C)	Oxygen saturation (%)
1	1	20	7.6 ± 0.1	105.7 ± 8
1	2	20	7.6 ± 0.1	103.1 ± 8
1	3	20	13.7 ± 0.2	99.2 ± 14
1	4	20	13.7 ± 0.2	95.1 ± 14

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. Personnel executing the procedures were trained in accordance with the FELASA C category.

3.3. Sampling of lumpsucker skin:

In the temperature acclimation experiment, a total 12 fishes were randomly collected from the cold and 12 fishes from warm acclimation (8 °C and 14 °C) after 7 days. Prior to skin collection, the fishes were anaesthetized using MS-222 (300 mg/l) and killed by a blow to the head. Immediately, skin samples were taken from lateral line in between the dorsal fin and the gill cover and were immediately frozen in liquid nitrogen and stored at - 80 ℃ freezer for later use.

3.4. Two-dimensional gel electrophoresis (2DE):

3.4.1. Preparation of skin sample for 2DE analysis:

For 2DE, protein samples from individual fish skin were extracted following the procedure of (Wang et al., 2007) and (Kulkarni et al., 2014). Skin samples were thawed on dry ice and macerated with mortar and pestle using liquid nitrogen to prevent degradation. Immediately, the macerated skin sample was resuspended into PBS containing 0.1 % protease inhibitor cocktail ((10 µl/ml) following the manufactures recommendation), then sonicated (2 x 5s) twice whilst on ice using an ultrasonic processor (SONICS Vibracell VCX750, USA). The mixture was centrifuged (Micro centrifuge- Heraeus™ Fresco™, Thermo Scientific) at 15,000×g for 30 min at 4 °C, then the supernatant was transferred to a new Eppendorf tube containing 10 % (w/v) TCA and 0.1 % DTT.

The mixture was maintained on ice for 30 min to precipitate, centrifuged at 10,000×g for 30 min at 4 °C, the supernatant was discarded. The precipitate containing the total protein in pellet form, was resuspended in cold acetone containing 0.1 % DTT and kept at -20 °C for 45 min and later centrifuged at 10,000×g for 30 min at 4 °C. The supernatant was discarded, the pellet air dried for 3 min and dissolved in rehydration buffer [9.8 M urea, 2 % CHAPS, 20 mM DTT, 0.5 % BioLyte and 0.002 % bromophenol blue] then slow vortex (Vortex 2 Genie, Scientific Industries, Inc.) for 30 min at room temperature to solubilise the protein and centrifuged at 10,000×g for 30 min at 15 ℃. The supernatant containing the soluble protein fraction were collected and 10 µl aliquots were to dialysed by using a 3K cutoff centrifugal filters (VWR, Norway) by following the manufactures protocol. Dialysis was made by using proteomic grade water thrice to remove salts. Protein obtained by the dialysis process was used to quantify the total protein content using Qubit® protein assay kit and the Qubit® flurometer (Qubit 3.0 Flurometer-Invitrogen, Life Technologies). The soluble protein sample was stored at -80 ℃.

3.4.2. Rehydration (passive) of proteins in 17 cm IPG strips:

The soluble protein fraction containing 100 µg of protein in 300 µl rehydration buffer was used to rehydrate 17 cm isoelectric strips (IPG strips, Bio-Rad) in a rehydration tray. The strip was carefully put in the rehydration tray by placing the gel side down above and the mineral oil added on top of the strip to prevent the sample from drying out. Later the tray was closed and kept undisturbed for 15-17 h at room temperature for passive rehydration.

3.4.3. Isoelectric focusing (IEF) of IPG strips:

In the IEF tray paper wicks (Bio-Rad) were placed on both the positive and negative electrode, 8 µl of proteome grade water was added to wet each wicks. Excess amount of mineral oil was drained from the passively rehydrated strip and they were placed on the IEF tray oriented carefully with respect to their positive and negative end and with gel side down. Mineral oil was added to the tray before starting the focusing run to prevent evaporation during focusing. The focusing tray was placed into the Protean IEF cell (Bio-Rad) and run following the procedure of (Rajan et al., 2011), for all IPG strips. In short, a constant temperature of 20 ℃ with a maximum current of 50 µA/IPG strip, subjecting the p*I* strips to a maximum voltage of 1×10^4 in three slow ramping steps to attain a total volt hours of 6×10^4 . After completion of the IEF run, the mineral oil was let to drip of the IPG strips and they were placed in -80 ℃ for later use or directly taken for equilibration.

3.4.4. Equilibration and SDS-PAGE:

Prior to running the second dimensional electrophoresis equilibration of the strips are necessary. The strips previously kept at -80 ℃ were thawed for 15 min. 2 % w/v DTT added to the pre-equilibration buffer (10 ml, 6 M urea, 0.375 M Tris-HCl (pH 8.8), 2 % SDS, 20 % glycerol) to prepare the pre-equilibration buffer I (EB -I) and 3 % w/v of iodoacetamide added to the pre-equilibration buffer to prepare 10 ml of EB-II. Equilibrate the IEF run IPG strips in EB-I for 15 min in a pipette sealed with para film on shaker, then in a new pipette equilibrate the strips in EB-II for 15 min. The equilibrated strips were subjected to a second dimensional electrophoresis in 12.5 % polyacrylamide gels using Bio-Rad Protean IIxii system (USA) by using bromophenol blue to track the gels. Initially, gels were run at 40 mA for 15 min then on 12 mA overnight. The next day, the voltage was increase to 30 mA to complete the electrophoresis. After completion of the run gels were stained by Sypro® Ruby using manufacture's protocol, the gels were fixed by immersing in the fixing solution (50 % methanol + 7 % acetic acid) and agitated on an orbital shaker (BioCote, UK) for 30 min. The fixed gels were stained in the Sypro® Ruby for overnight under agitation in the dark (covered with aluminium foil). Following day, the gels were washed using washing solution (10 % methanol + 7 % acetic acid) for 30 min. The gels were further washed in 15Ω analytic grade Millipore water and agitated for 5 min, then visualised the gels in UV transilluminator mode in the ChemiDOC^{TM} XRS imaging system (Bio-Rad). The documented gels were analysed using PDQuestTM Advanced 2D analysis software 8.0.1 (Bio-Rad). Next, the selected spots from the analysis were excised manually on a blue light transilluminator (Safe Imager™ 2.0 Blue- Light Transilluminator, Life technologies, USA) and subjected for LC-MS/MS analysis.

3.5. Statistical analysis of gels:

The 2DE gels were analysed for spot detection, to identify the significant spots, the spot normalization was performed by local regression model in the PDQuest software. Student's t-test (95 % confidence interval) was employed in the statistical analysis to determine any significant differences in spot intensity ($p<0.05$). The confidence limit for detecting meaningful appearance or disappearance of spots was defined as 10-fold of the background level (recommended by the manufacturer). For the following reproducibility tests for up- and down-regulation of protein spots, the confidence threshold for differential expression was set at 1.5 and 2-fold below or above the spot intensity. Further, data analysed for non -parametric test by using KS normality test (Kolmogorov-Smirnov Test) in Graphpad Prism 7 and applied two paired 't' test, mean ± SD performed for acclimated group using excel software (Microsoft).

3.6. LC-MSMS analysis:

The identified peak protein spots were analysed by Department of Medical Biology, University of Tromsø. Protein band/ spots were in-gel reduced, alkylated and trypsinised as described elsewhere (Øverbye et al., 2007). The peptide mixture was subjected to LC-MS/MS analysis using ESI-Quad-TOF, USA. Peak lists were obtained by Protein Lynx Global server software (version 2.1) and converted to mascot generic file (mgf). The mgf files were analysed for the protein identification using appropriate bioinformatics tools.

3.7. Bioinformatic analysis:

Mascot generic files obtained from LC-MS/MS analysis were subjected to the MASCOT [http://www.matrixscience.com] search engine and searched against NCBIprot, SWISSprot and Actinopterygii EST with the following parameters: maximum one missed cleavage by trypsin, peptide mass tolerance 100 ppm, MS/MS ion tolerance set to 0.1 Da, fixed carbamidomethyl modification and variable oxidation modification.

3.8. Isolation of lumpsucker skin proteins:

3.8.1. Sampling of lumpsucker skin and mucus:

Hatchery reared lumpsucker was brought to Mørkvedbukta Research Station, (WGS84: 67°16'41.7"N 14°33'26.8"E) Nord University, from Arctic Cleanerfish in Stamsund, Lofoten (WGS84: 68°7'5.0"N 13°47'14.2"E) and was maintained described in section 3.1. Ten fishes, with an average weight of 141± 20 g were used for the skin samples. For the mucus collection fishes reared upto an average weight of 4 kg was used (Fig. 1). Prior to skin collection, the fishes were anaesthetized using MS-222 (300 mg/l) and killed by a blow to the head. Skin samples were taken from the lumpsucker by peeling the skin from the fish and immediately frozen in liquid nitrogen. In the skin mucus collection, fish were kept on a plastic bag and massaged gently for a few seconds, then by using sterilised slides mucus was gently scrapped from the skin and by avoiding contamination of feces. The mucus was transferred into tubes and both skin and mucus was stored at -80 ℃ for later use.

3.8.2 Preparation of skin sample for isolation:

Skin samples stored at -80 °C were taken out and thawed on dry ice, pulverised by using pestle and mortar in liquid nitogen and then diluted 1:10 with binding buffer (20 mM Tris- HCl, pH 7.5, 0.5 M NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$) in the presence of protease inhibitor (10µl/ml) following the manufactures recommendation. These mixtures were sonicated twice for 5s on ice

using ultrasonic processor (SONICS Vibracell VCX750, USA). The diluted skin mucus was centrifuged 15,000 × g to remove debris and the supernatant was used for the purification step.

3.8.3. Isolation of mannose binding proteins from skin:

Purification was done by following the protocol established for Atlantic cod skin mucus described by Rajan et al., 2013 with a few modifications. For the present work, 10 ml of mannose sepharose resins in a 50 ml tube was washed twice with 3 volumes of 15 Ω analytic grade Millipore water at 4 °C. The washed resin was then equilibrated with 3 volumes of binding buffer (section 3.7.1) at 4 °C. To the equilibrated resin, skin mucosal extract (15 ml, from section 3.7.1) was added and was incubated to bind the sample to the resin for 2 h at 4 °C under rotation (VWR tube rotator). The bound skin mucus was washed 3 times with binding buffer. To elute the bound mannose binding proteins in the resin, elution buffer (binding buffer with 0.5 M mannose) was used under rotation for 1 h at 4 °C. For the fraction collection, the resin with elution buffer was transferred carefully to a gravity purification column (Econo Column 1.5 cm × 15 cm Bio-Rad, 27 ml capacity) and twenty fractions, each of 500 µl eluate were collected and the protein was used for quantification. The column was washed twice using binding buffer and stored in 4 °C for later use. Eluted fractions containing protein were up-concentrated using 3K cut off centrifugal filters and kept for later use. The protein quantification was done by using Qubit® Protein Assay Kit in a Qubit fluorometer.

3.8.4. SDS-PAGE:

Mannose binding protein fractions was subjected to the SDS-PAGE to determine the number of proteins and their molecular weights. Gels were prepared by using 15 % resolving and stacking 4 % stacking gels (Laemmli 1970). Samples were thawed on ice, mixed in 1:1 ratio with protein loading buffer (Laemmli buffer mixed with β-mercaptoethanol) and incubated at 95 °C on a heat block (AccuBlock™ Digital Dry Baths) for 5 min. A protein standard marker to determine molecular weight (range:10-250 kDa, Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards) was loaded in one lane of the gel and samples in the remaining lanes. The gel was run using SDS running buffer in the Mini Protean cells (Bio-Rad) at 100 Volt for 2 hr. Gels were stained in colloidal Coomasie (G-250) overnight, then next day, the gel was destained using 15 Ω analytic grade Millipore water and images were captured using the ChemiDOC^{TM} XRS imaging system.

3.9. Hemagglutination assay:

Hemagglutination activity was measured by using defibrinated horse erythrocyte (Thermo-scientific). Erythrocytes were treated in trypsin in and fixed by glutaraldehyde, diluted to 4 % erythrocyte solution as reported earlier (Nowak et al., 1977) with some modification. In short, TBS I buffer (Tris buffered saline; 20 mM Tris-HCl,150 mM NaCl, pH-7.6) was used to initially wash 3 times until erythrocyte in buffer solution became clear, then they were treated with 0.1 % trypsin EDTA in TBS-I for 1 h in room temperature and extensively washed in TBS-I. Further, erythrocytes were fixed in 1 % glutaraldehyde in TBS-I for 1 h. Later, fixed erythrocytes were washed twice in 0.1 M glycine in TBS-I and again washed extensively in TBS and diluted to 4 % erythrocyte concentration.

The hemagglutination assay was performed in U bottomed microtiter plates (VWR, Norway). To test the activity, sample was dispended in each test well in the following scheme, 25 µl of protein sample (1 mg/ml), 25 µl of erythrocyte suspension, 25 µl of 0.5 % BSA-TBS I and 25 µl TBS II (TBS I + 10 mM $CaCl_2$, pH-7.6) for calcium dependent protein and to check independent activity 10 mM EDTA in TBS I (pH-7.6). To find the hemagglutination titre, 2-fold serial dilutions of the protein sample were employed. In control wells, the protein sample was replaced with 25 µl TBS II. For hemagglutination inhibition assays, TBS I was replaced with 25 µl of 0.5 M mannose in TBS I.

For the microscopic visualisation of hemagglutination, isolated proteins were dialysed for 12 h in EDTA-TBS I and after EDTA, then, further dialysed in cations TBS-II and TBS-Mg^{++} for 12 h. Sterilised slides were prepared by adding 10µl of dialysed protein and mixing with 2 µl of 4 % erythrocyte in TBS-I (without fixation) without dilution in BSA and TBS buffer slides. The hemagglutination activity were observed under microscope (Leica DM 400 B, Leica Microsystems) after 5 min before drying the solution in slide.

3.10. Bacterial agglutination:

A stock culture of bacterial strain *Aeromonas salmonicida* (NCIMB 1102) (Sirriyappagouder, 2013) stored at -80 ℃ was used for the agglutination test. The bacteria were cultured in 3 % TSB with 1.5 % NaCl at 15 ℃. Absorbance of culture was measured at 600 nm in a spectrophotometer (Genesys 10s UV-Vis, Thermo-Scientific) and when the growth reached to 0.6 OD (optical density), it was used for the agglutination test.

In the agglutination test, bacterial cells were adjusted to 1.2 $\times 10^8$ cells (using TSA plate count method) based on the OD at 600 nm washed in TBS, fixed in 4 % paraformaldehyde at 4 ℃ for 16

h, washed again in TBS I thrice and labelled with DAPI for 10 min at room temperature. DAPI labelled bacterial cells suspended in TBS and mixed 15 µg of the purified proteins with equal volume of bacterial cells in TBS II and TBS-I, controls were with skin extract, mucus and 0.1 M mannose in TBS used to check the bacterial activity.

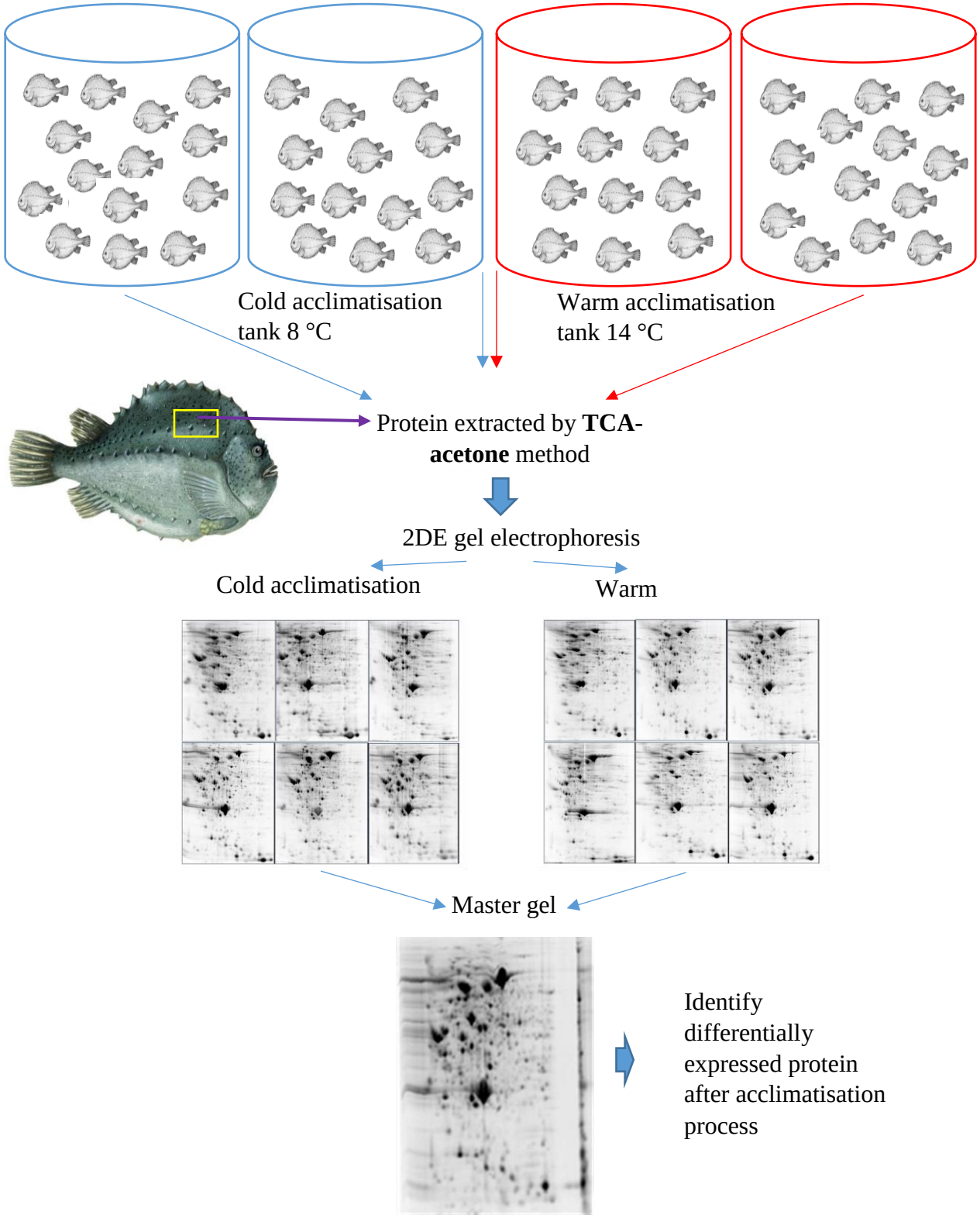
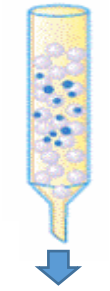


Figure 2a. Experimental setup for temperature acclimation

Skin tissues

Tissues pulverised using liquid nitrogen in pestle and mortar



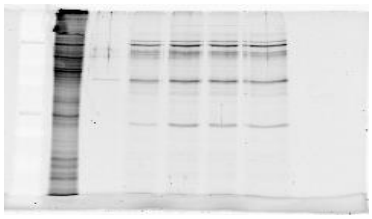
Pulverised tissues in binding buffer at 4°C using **carbohydrate affinity column**

Eluted using elution

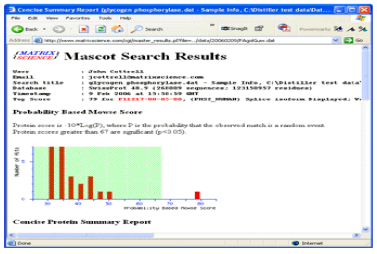
isolated protein fraction

Protein identity

SDS-PAGE

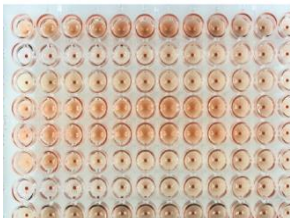


Mascot results for the above identified bands using LC-MS/MS



Characterisation

Hemagglutination and agglutination



Bacterial agglutination

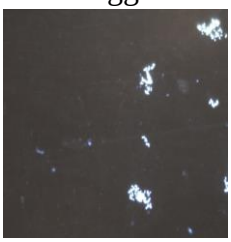


Figure 2b. Experimental set up for the isolation of mannose binding proteins from the skin in mannose sepharose affinity

4. Results

4.1. 2DE analysis on lumpsucker acclimation:

Two-dimensional electrophoresis with p*I* 3-10 gel strips in the first dimension and 12.5 % SDS PAGE gels as the second dimension was performed for on skin samples. Lumpsucker 2DE gels (Appendix III) were analysed for both high temperature acclimation 14 °C (n=6) and cold acclimation 8 °C (n=6) by using PDQuest software advanced (Bio-Rad, USA). All the 12 gels showed ≈500 spots and the master gel (≈ 600) spots identified by the software. A representative gel image is shown in Fig. 3 and all gels are shown in miniature in Fig. 2a. The variation in protein spot density between the acclimatised groups were calculated as fold ratios, 17 spots were highly expressed in the control or warm acclimated with a fold change of 1.5 (up regulated ≥1.5 and down regulated ≤ 0.66) and spots were excised for LC-MS/MS analysis. Among 17 spots 5 spots were not identified in the MASCOT analysis the rest of the proteins were identified by adapting homology searches in MASCOT restricting the searches to the class Actinopterygii (ray finned fishes). The MASCOT summary of results for each identified spot is mentioned in Appendix I.

Table 4. Protein names and parameters obtained from MASCOT search of differentially expressed proteins from the lumpsucker acclimation are listed below

No	Protein Name	Acc. No	Theoretical p*I*/Mr	IS	SC (%)	FCx
1	Small ubiquitin-related modifier 2 *Notothenia coriiceps*	XP_010770112.1	5.32/10885	58	12	0.18
2	Not identified					
3	Prefoldin subunit 5 *Larimichthys crocea*	KKF32533.1	9.30/14604	66	13	0.36
4	Not identified					
5	SH3 domain-binding glutamic acid-rich-like protein *Osmerus mordax*	ACO10145.1	4.78/ 13128	153	21	0.42
6	Protein mago nashi homolog *Danio rerio*	NP_001017700.1	5.95 /17269	45	7	0.45
7	Not identified					

8	Proteasome subunit beta type 1-A *Osmerus mordax*	ACO09437.1	7.00/26440	208	15	0.66
9	Retinoblastoma binding protein 4, like *Danio rerio*	AAI54779.1	4.71/48095	168	6	1.51
10	Angiotensinogen-like *Neolamprologus brichardi*	XP_006783765.1	5.19/52208	210	5	2
11	Microfibril-associated glycoprotein 4-like *Cyprinus carpio*	XP_018936831.1	7.51/ 28240	61	4	2.09
12	Apolipoprotein E *Oplegnathus fasciatus*	ACF21982.1	4.93/31040	191	20	2.19
13	Not identified					
14	40S ribosomal protein *Salmo salar*	NP_001134397.1	4.93/ 35043	118	20	2.36
15	Annexin A3 *Larimichthys crocea*	KKF17944.1	5.19/ 36677	136	5	2.58
16	Catechol O-methyltransferase domain-containing protein 1-like, *Notothenia coriiceps*	XP_010780849.1	5.45/ 27212	181	14	2.76
17	Not identified					

*Acc. No-Accession number; p*I*/Mr (molecular mass); IS (ion score); SC-sequence coverage; FCx- fold change

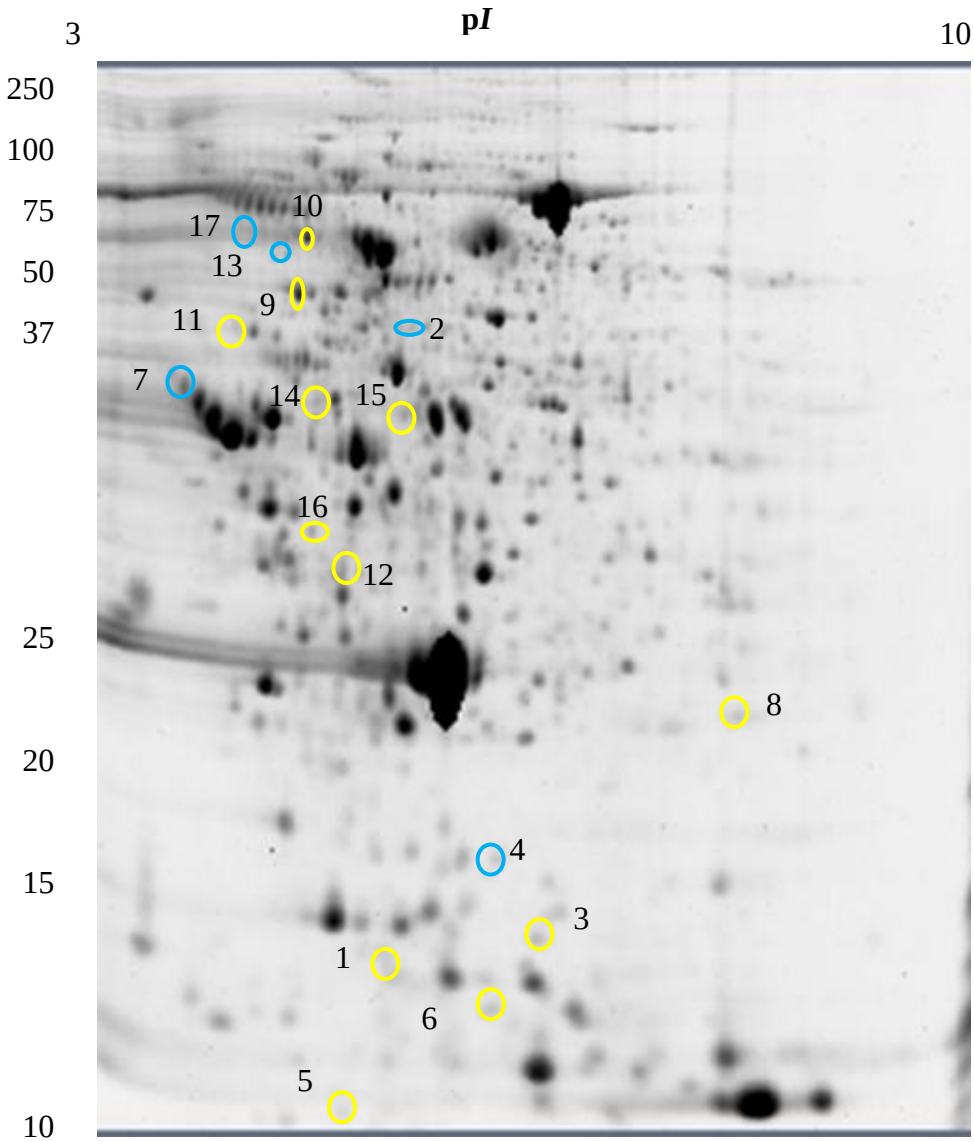


Figure 3. Two-dimensional gel image for acclimation experiment; spots marked in the gel are differentially expressed spots, spots with yellow circles were identified, spots in blue circles are not identified spots.

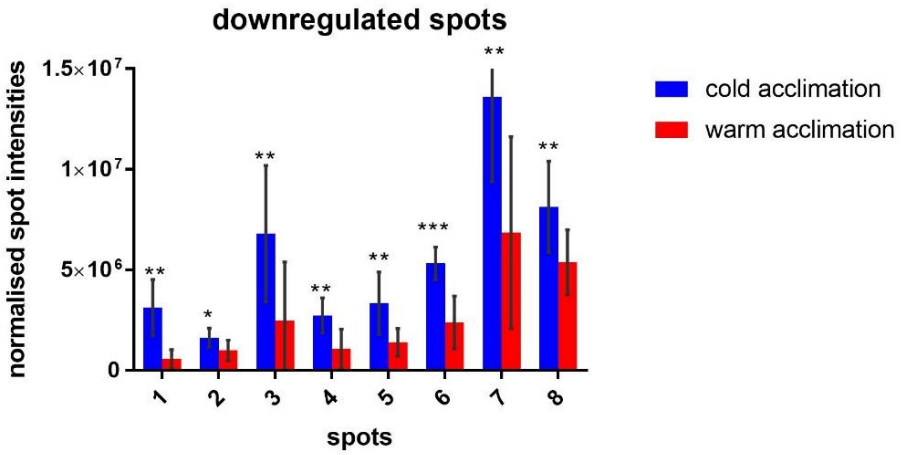


Figure 3a.

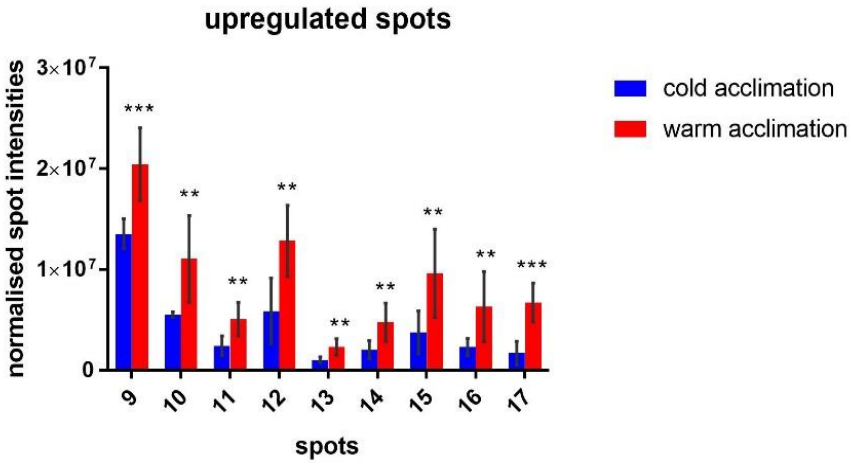


Figure 3b.

Figure 3a & 3b. Normalized spot intensities of differentially expressed proteins (p<0.05) based on PDQuest student's t-test and non-parametric Kolmogorov-Smirnov test (Graphpad Prism 7) used to evaluate the difference between the cold vs warm acclimation in lumpsucker, N=6; The means ± S.D were calculated by Excel software (Microsoft). (in graph: *p< 0.05, **p<0.01, ***p<0.001)

4.2. Isolated mannose binding proteins from lumpsucker skin:

To isolated mannose binding proteins from lumpsucker skin a mannose sepharose affinity column was used. The elute from the column showed five bands (Fig. 4a) and sephacryl S 200 HR (Fig.4b) gel filtration showed 6 bands, the bands had a molecular weight of band 1 obtained in the SDS-PAGE was above 76 kDa, band 2 was at 75 kDa and band(s) 3(3a and 3b) in between 75 and 50 kDa, band 4 approximately 38 kDa and last band was observed approximately 24 kDa were determined in the polyacrylamide gel.

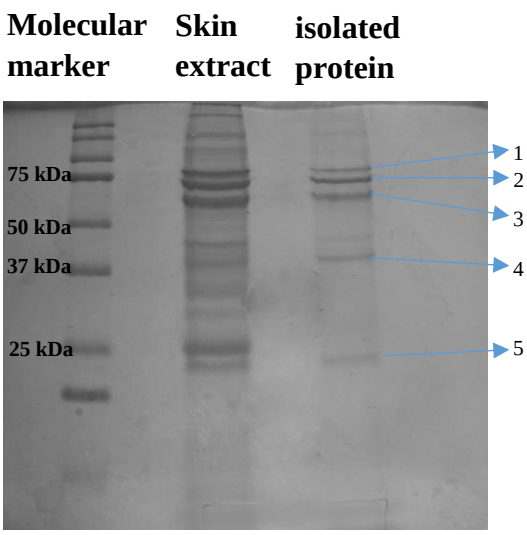


Figure 4a. SDS-PAGE of mannose sepharose isolated lumpsucker skin proteins. Affinity-purified lectin was subjected to SDS-PAGE on a 15 % gel.

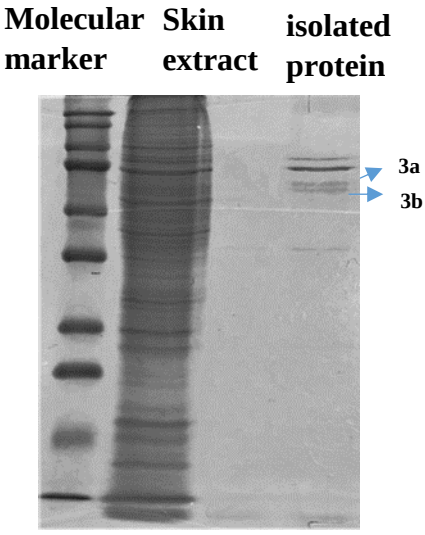


Figure 4b. SDS- PAGE of sephacryl S 200 HR. Gel filtration of isolated proteins on a 15 % gel

4.2.2. Identification of isolated bands:

The isolated bands (Fig. 4a & 4b) were excised and analysed by mass spectrometry on the ESI-Q-TOF instrument, the result files obtained were analyzed using MASCOT (http://www.matrixscience.com/). The analysis in mascot database identified the protein as shown in the Table 5.

Table 5. Protein name and parameters obtained from MASCOT search for mannose binding proteins

No.	Protein name	Acc. No.	Theoretical pI/Mr	SC (%)	IS
1.	Not identified				
2.	Transferrin *Trachidermus fasciatus*	AEV21971.1	5.80/ 75092	3	149
3a.	Glial fibrillary acidic protein *Carassius auratus*	P48677.2	4.93/42636	3	83
3b.	a1-antitrypsin *Monopterus albus*	AMQ75915.1	6.52/46723	3	55

4.	Fucose binding lectin *Trachidermus fasciatus*	GI:530695833	5.75/34580	2	56
5.	Pentraxin fusion protein-like *Sinocyclocheilus grahami*	XP_016093745.1	5.96/25478	8	127

*Acc. No-Accession number; p*I*/Mr (molecular mass); IS (ion score); SC-sequence coverage

4.3. Hemagglutination assay:

Hemagglutination was observed for both skin (Fig. 4) and mucus (gel in Appendix IV) isolated mannose binding proteins in the presence of $CaCl_2$. Hemagglutination was not observed with a lower concentration but was only successful with a higher concentration of 1 mg/ml protein with 4 % horse erythrocytes in the presence of TBS-II, BSA Fig. 5 (**A_1** and **A_2**) and to check calcium dependence of the isolated proteins EDTA in TBS-II was used and partial inhibition was observed in **B_1, B_2** and **C_3** Fig. 5.

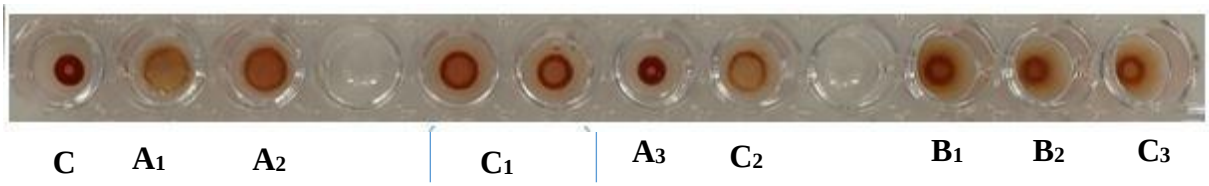


Figure 5. Hemagglutination assay of the isolated mannose binding proteins; (+) uniform mat covering the bottom of well with isolated mannose binding proteins (**A_1**); (+) less covering mat surrounded by dense ring (**A_2**, **C_1** & **C_2**); (+/-) dense ring with hazy edge with shadow mat (**B_1**, **B_2** & **C_3**) negative control and mannose as dense red ring with clear edges (**C** & **A_3**) (Halo negative).

C- negative control (TBS-II with BSA and 4 % erythrocyte)

A_1 and **A_2** - skin and mucus isolated proteins (TBS-II with BSA and 4 % erythrocyte)

C_1 – both skin extract and mucus in binding buffer (TBS-I with BSA and 4 % erythrocyte)

A_3 - 0.1 M mannose in TBS-I with BSA and 4 % erythrocyte

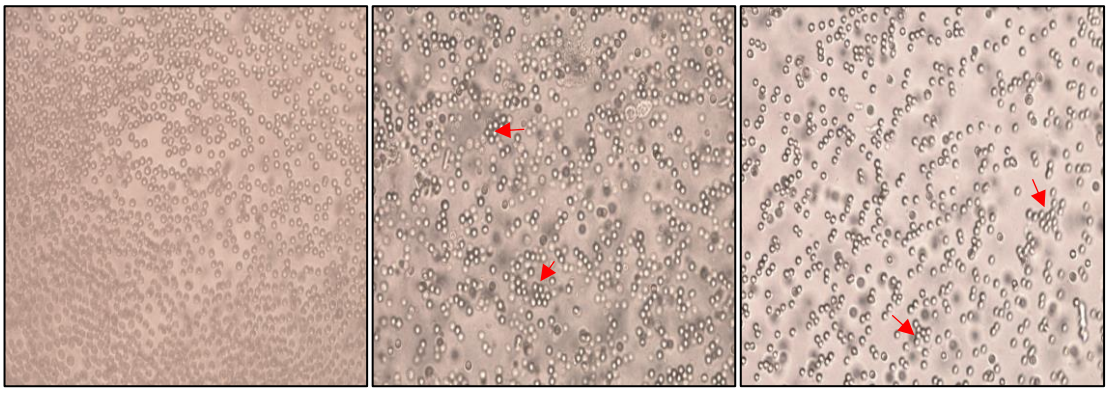
C_2 – positive control mucus in TBS-I with BSA and 4 % erythrocyte

B_1 and **B_2** – skin and mucus isolated proteins in TBS-II EDTA with BSA and 4 % erythrocyte

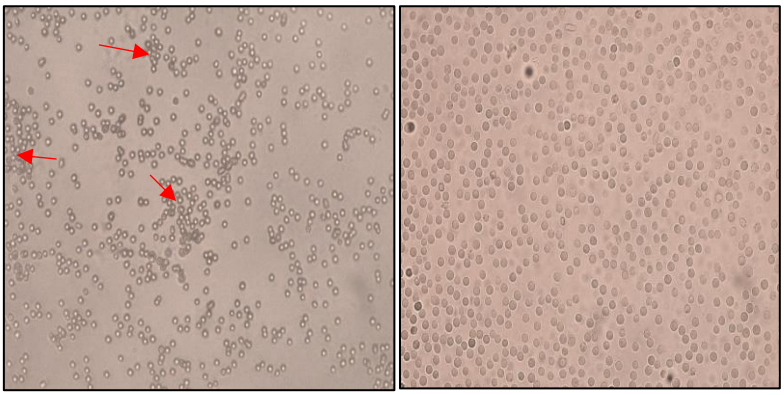
C_3- negative control for TBS-11 EDTA with BSA and 4 % erythrocyte

4.3.1. Hemagglutination assay under microscope:

The isolated mannose binding proteins at 250 µg/ml concentration after 12 h dialysed in EDTA and 12 h in Ca^{++} were effectively hemagglutinated to 4 % erythrocytes in microslide and observed under microscope (40x magnification) using phase contrast. As shown in Fig.6 erythrocytes clump and stick together (red arrows in Fig. 6) in mucus (**M)**, skin **(S),** isolated mannose binding proteins (**A)** and erythrocytes were compact but not clumping in control (**C_1**) or with isolated mannose binding proteins in Mg^{++}(**B**). All the interaction was observed within 5 min of incubation in the microslide.



C_1 **M** **S**



A **B**

Figure 6. Hemagglutination activity of isolated mannose binding protein under microscope; **C_1**- 4 % erythrocytes in TBS-II; **M**-lumpsucker mucus with 4 % erythrocyte; **S**- skin extracts in binding buffer with 4 % erythrocytes; **A**- isolated mannose binding proteins in TBS-II; **B**- isolated mannose binding proteins in TBS-Mg^{++}

4.4 Bacterial agglutination assay:

Isolated mannose binding proteins agglutinated 4 % paraformaldehyde fixed DAPI stained *A. salmonicida* in the presence and absence of calcium (Ca^{++}) as observed under fluorescent microscope (40x magnification, Fig. 7). Mucus and skin extracts were also agglutinating the bacteria (Fig.7). Bacterial cells were clumped together in the presence of agglutination as whitish patches (yellow arrows) in Fig. 7: **c, d, e** and **f** and in the absence of agglutination the bacterial cells were scattered freely in **a** and **b.**

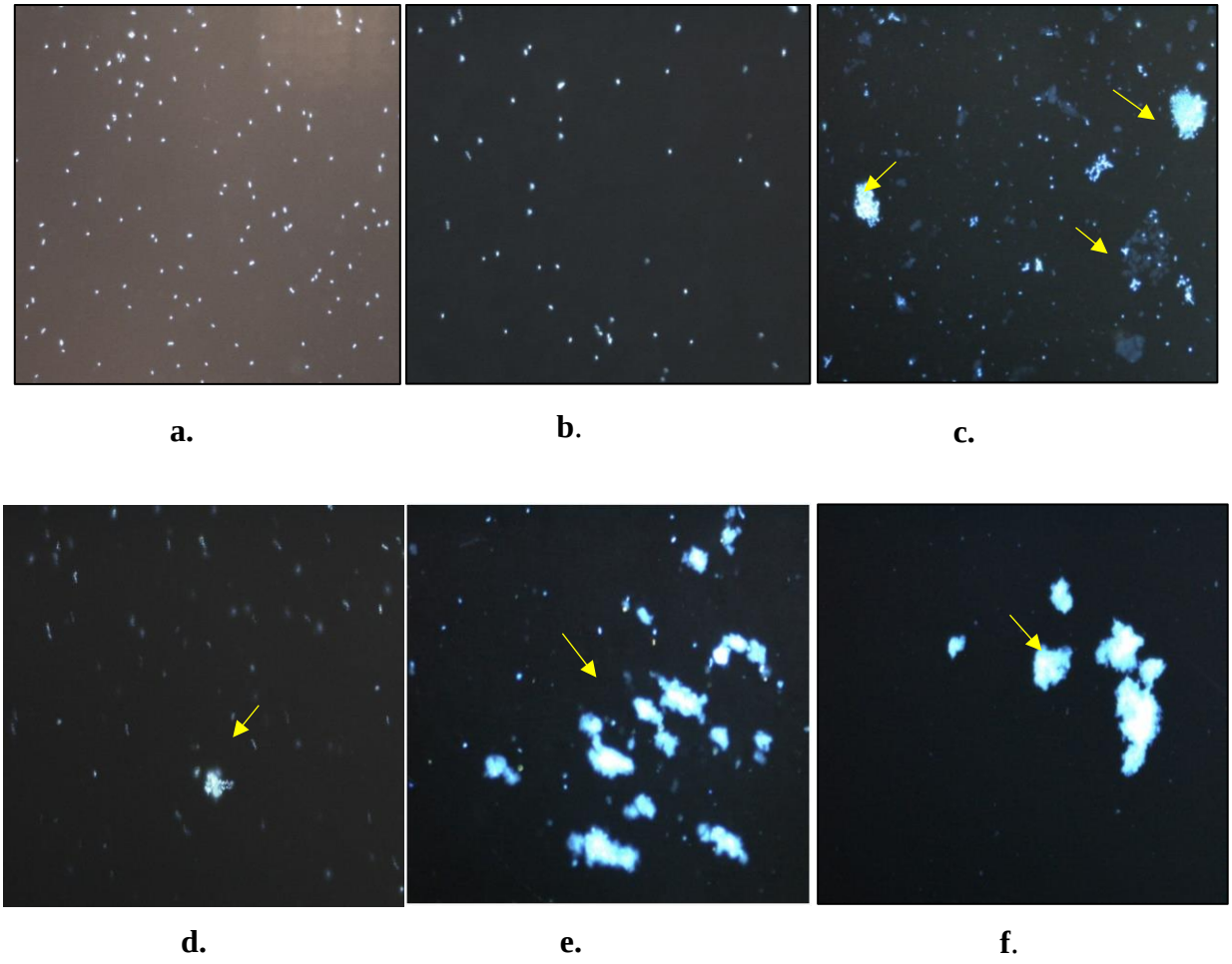


Figure 7. Agglutination of *A. salmonicida*; **a-** control bacteria in TBS-II; **b-** control bacteria in mannose; **c-** mucus agglutination; **d-** agglutination by isolated skin proteins without Ca^{++}; **e-** agglutination by isolated skin proteins with Ca^{++}; **f-** agglutination by skin extract in binding buffer. Yellow arrows points to agglutinated bacterial cells.

5. Discussion:

The skin in the fishes is considered as a barrier against biotic and abiotic factors, which helps to secure fish integrity. It was observed during the study that lumpsucker skin was rough and scaleless and had less mucus production to the exterior compared with other fishes. For this reason and because little information currently available on lumpsucker skin, in the present study skin was used instead of mucus (Fig. 1). We identified stress proteins that are functionally important for the cellular function and innate immunology mechanism. The expression of identified proteins in temperature acclimation may help the fish to compete the new higher temperature. Characterisation of mannose binding proteins in the skin in lumpsucker was done to understand importance of mannose proteins in innate immunity. In the stress situation, fishes may be most vulnerable to infection, this study also proteins that might be immunological important to lumpsucker for its welfare issues in the salmon farming system during warmer temperature.

5.1. Temperature acclimated lumpsucker:

In the present study, lumpsucker was acclimatised to 14 °C and compared with fish kept at cold seawater for 8 °C. It was to investigate the stress related proteins at proteomic level after the temperature acclimation for a week. Recently temperature increases are noticed in the Norwegian sea and new species introduced to the salmon farming is challenging, particularly in the summer, some of the western and southern regions of Norway experience temperature increments above the thermal preference (15 °C) (Hevrøy et al., 2013) for prolonged periods. The acclimation study was conducted to check the temperature stress in the fishes, no mortalities were noted during acclimation. In the study acute stress was conducted to check the modification of the protein after acclimation and also to understand the physiological state and studies also reveal that (Logan and Somero, 2010) long-term acclimation to increased temperature, there was no upregulation of stress-related proteins and little variations, differences in expression of genes involved in protein biosynthesis, transport and various metabolic categories. In the present study, acclimation temperature to target 14 °C in 48 h and continued for one week and in the case of rainbow fish, they were acclimatised for six days to reach 33 °C and continued for 14 days and genes involved in metabolic pathways (Smith et al., 2013). In the present study, differential expression of proteins was observed between fishes kept at 8 °C and fish where the temperature was upregulated to 14 °C for a total of 7 days.

5.1.1. Spots identified from MASCOT analysis:

Spots in the gels with significant difference between acclimatised group were identified by using MASCOT. Among 17 spot with differential expression (Fig. 3a and 3b), with respect to their

acclimation temperature, five were not identified in MASCOT. Spots obtained in the present study may be involved in various heat stress coping pathways. Gene expression of many of the same molecules were found to be differentially expressed in catfish gill and liver (Liu et al., 2013) and grouped into six functional categories protein folding, degradation, biosynthesis, molecule and ion transport, energy metabolism and cytoskeletal reorganisation. Some of the identified molecules in our study were found in the skin mucus of chronical stressed gilthead sea bream (*Sparus aurata* L.) (Pérez-Sánchez et al., 2017). In our study identified protein's function related to stress may be in protein folding (PFDN-5); protein degradation (PSMB1, SUMO-2), protein biosynthesis (MAGOH, SH3BGRL3, RPS, RBBP4, AGT); energy metabolism (APO-E, COMTD1), cytoskeletal reorganisation (MFAP4) and cell proliferation and apoptosis (ANXA3) (Tacchi et al., 2012).

Downregulated spots:

Small ubiquitin-related modifier 2 (SUMO-2): An important cellular component involved in many cellular processes. In human, SUMO-1/2/3 proteins were shown to be localized on nuclear membrane, nuclear bodies and cytoplasm respectively (Su and Li, 2002). In the present study, SUMO-2 was identified in the skin. SUMO in the grouper (*Epinephelus coioides*) EcSUMO1 and EcSUMO2 were identified intracellularly (Xu et al., 2016), highly expressed in intestine and gill and responded to viral infection with Singapore grouper iridovirus (SGIV). In temperature acclimated fishes, the decreased spot intensity was found.

SH3 domain-binding glutamic acid-rich-like protein (SH3BGRL): The redox proteins SH3BGRL was identified in our study, it was downregulated in the warm temperature acclimation. In the infected salmon these proteins were downregulated in the gill mucus (Valdenegro-Vega et al., 2014). In the proteome map of the European seabass a similar protein was observed (Cordero et al., 2015). The gene expression profiles of *Trematomus bernacchii* in the 7 d multi-stressor treatment showed upregulation of redox protein SH3BGRL3 (Huth and Place, 2016). In the present study, expression was downregulated in warm acclimation. The mechanism behind this is not clear. The oxygen saturation in the water throughout the experiment period was same (Table. 3), 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 (Kammer et al., 2011, Lushchak, 2011).

Protein mago nashi homolog (MAGOH): Mago nashi protein is involved in splicing, transport of mRNAs and deciding mRNA subcellular location (Le Hir et al., 2001). Mago nashi homolog is involved in transcription and translation process. In a RNAseq study of catfish temperature stressed for up to 4 days, protein mago nashi transcripts were downregulated both in liver and gills (Liu et al., 2013) as was the protein in this lumsucker study. The mago nashi protein homolog were reported to be downregulated in *Penicillium glabrum* subjected to thermal stress (Nevarez et al., 2008).

Proteasome subunit beta type 1-A (PSMB1): Cellular component present in the cytoplasm and a sub unit of the proteasome a major protein degradation site in cells. Information of proteasome subunit beta type, in the fish is scarce, however proteasome such as alpha subunit has been observed in the mucus of naïve Atlantic cod (Rajan et al., 2011) and proteasome 26S subunit levels are significantly upregulated after the infection Atlantic cod with *V. anguillarum* (Rajan et al., 2013b). Cellular degradation activation in muscle was compared in the annual fish (*Nothobranchius rachovii*) exposed to three temperatures: above (30 °C) and below (20 °C) and the normal (25 °C) for 45 days. Low temperature induces high 20S proteasome activity, whereas high temperature inhibits 20S proteasome activity (Lu and Hsu, 2015). In lumpsucker similar observation in the skin from low temperature for 7 days of acclimation with high spot intensity and less intensity in warmer temperature and this indicate that the ATP-dependent proteolysis way was repressed inorder to minimise the heat stress and similar observation observed in catfish upon heat stress (Liu et al., 2013).

Prefoldin subunit 5 (PFDN5): Co-chaperon**,** cytosolic, involved in the process of proper folding. Prefoldin binds specifically to cytosolic nascent polypeptides and promotes folding, it is a highly specialized co-chaperone for actin and tubulin folding. Prefoldin co-chaperon mediates transfer of newly synthesized proteins from HSP complexes to the cytosolic chaperonin (Young et al., 2004). Prefoldin is composed of six different subunits with molecular masses in the range 14–23 kDa (Vainberg et al., 1998) and the protein spot we identified showed a molecular mass of 14 kDa and spot intensity was reduced in warm acclimation compared to cold. In contrast to this in the heat stress of coral reef fish *Pomacentrus moluccensis* from 28 ℃ to 31 ℃ prefoldin subunit 4 was highly expressed (Kassahn, 2006).

Upregulated spots:

Retinoblastoma binding protein 4, like (RBBP4): Regulates chromatin metabolism, promote histone deacetylation and transcriptional repression. Another RBBP, protein subunit RBBP 2 act as an antisense protein for the hypoxic condition in euryoxic fish (*Gillichthys mirabilis*)

upregulation was observed strongly after 48 h in liver, skeletal muscle (Gracey et al., 2001). Histone-binding protein RBBP4 was upregulated in the muscle regeneration in trout (Montfort et al., 2016). The upregulation in acclimatised fishes might be controlling the cell proliferation in the skin. In the study of larval gnotobiotic seabass (*Dicentrarchus labrax*) histone-binding protein rbbp4 were upregulated when exposed to the probiotic candidate *Vibrio lentus* and involved in controlling cell proliferation (Schaeck et al., 2017). The RBBP4 involved in the protein synthesis and translation according KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway and in zebra fish when challenged with acute metal exposures upregulation was observed (Hussainzada et al., 2014).

Angiotensinogen-like (AGT): AGT is the precursor of angiotensin. Renin-angiotensin (RAS) play role in regulating blood and fluid balance, moreover, recent research also revealed that oxidative stress one of the cause for hypertension (Bagatini et al., 2011). Angiotensin-1 converting enzyme (ACE) play role in regulation of blood pressure via renin-angiotensin and the kinin-kallikrein systems (Ghanbari et al., 2015). In the salmon, upregulation of angiotensinogen was observed when injected with cortisol (Krasnov et al., 2012). AGT was also observed in the chronically stressed skin mucus of gilthead seabream (*Sparus aurata*) which were involved in acute phage response signalling pathway (Pérez-Sánchez et al., 2017).

Microfibril-associated glycoprotein 4-like (MFAP4): 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 (Romero et al., 2011) and MFAP family members are found universally in vertebrates and invertebrates, in humans observed in extracellular matrix. A previous studies showed differential expression observed for MFAP4 genes to different bacterial challenges in catfish (Peatman et al., 2008, Li et al., 2013, Sun et al., 2012). MFAP4 gene expressed brain, gill, head kidney, heart, liver, stomach, intestine, spleen, trunk kidney, skin and muscle (Niu et al., 2011) and in the present study, observed in skin, may be it is upregulated to contribute to repair or modification of tissues during stress. Further studies are needed to establish the exact role of MFAP4 in the stress condition of fish.

Apolipoprotein E (APO-E): Apo-E a lipid binding protein a transporter of cholesterol and 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 (Schneider et al., 1997, St Clair and Beisiegel, 1997). APO-E upregulation was reported in the cold tolerant (0.7±0.05 ℃) fishes when exposed to 18 h in olive flounders (*Paralichthys olivaceus*) and declined in the cold sensitive fishes (Hu et al., 2014) and upregulation of apolipoprotein also observed in zebrafish, (Scott and

Johnston, 2012) 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 (Zhang et al., 2017). The role of apolipoproteins is not only for the lipid homeostasis but equally important for their antibacterial activity for microorganisms in the mucus of the fish (Concha et al., 2003). The cholesterol transport might increase in warm tolerance in lumpsucker skin to stabilizing lipid fluidity by the enrichment of cholesterol in cell membranes.

40S ribosomal protein (RPS): 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 (Quinn et al., 2011). Ribosomal proteins have been recognised as biomarker for cellular stress (Pytharopoulou et al., 2008) and indicating that biogenesis occurred to the heat exposure (Quinn et al., 2011). Ribosomal proteins are involved 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 (Pérez-Sánchez et al., 2017).

Catechol O-methyltransferase (COMT) domain-containing protein 1-like (COMTD1): Protein involved in the O-methyltransferase activity and COMTD1 function in the skin of the fishes not established. The COMTD1 observed in the zebra fish larvae and deceased in response to a steroid treatment ($1\alpha,25(OH)_2D_3$) for 7 days (Craig et al., 2012). In the crimson spotted rainbowfish COMTD1 was downregulated in the liver of fish acclimated temperature of 33 ℃ for 14 days (Smith et al., 2013). In higher animals, present in soluble and membrane-bound forms. COMT is well known for salinity stress gene found upregulated in crustacean when exposed to low salinity (Rajesh et al., 2012). The COMTD1 were expressed differentially to *Flavobacterium psychrophilum* challenge in rainbow trout (Marancik et al., 2015).

Annexin A3 (ANXA3): Annexin belong to calcium dependent large protein family connected to cell membrane phospholipids basically involved in cellular functions, like membrane trafficking, exocytosis, endocytosis, membrane-cytoskeleton interactions, regulation of membrane protein activities, calcium channel activity (Lizarbe et al., 2013). Annexin proteins which are involved in the signal transduction in the cell have been observed in the early development stage of zebra fish (Ozerova and Minin, 2008). Annexin A3 is expressed in the endothelial lineages of fish, frog and mice, also it is dominant at embryonic endothelium stage in frog and fish compare to other annexin family members (Meadows and Cleaver, 2015). Anti-inflammatory activity of annexins (ANXAs) is attributed mainly to their ability to interfere with neutrophil extravasation (Perretti and Flower, 2004). Six annexins (A1, A2, A4, A5, A6 and A11) were upregulated in the channel catfish in the

early infection of *Edwardsiella ictaluri (*Yeh and Klesius, 2010). In the present study annexin A3 in the skin was upregulated in the warm acclimation temperature. In salmon, vaccination against furunculous annexin A1 (ANXA1) and annexin A3 (ANXA3) showed lower levels in vaccinated high resistance fishes (Škugor et al., 2009).

In the study, the differentially expressed spots might be involved in the stress response to the temperature acclimation. Proteins SUMO-2 and proteasome subunit beta type 1-A as ubiquitin/proteasome pathway 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. Both the ubiquitin/proteasome pathway were higher in higher temperature stress in the chinook salmon to 21 ℃ and 25 ℃ (Tomalty et al., 2015). 40S ribosomes binding protein might be involved in the acute response, by activating p53 pathway and plays an essential role in monitoring the balance between cellular growth and proliferation. Angiotensinogen expressed high in warm acclimation due to elevated blood pressure in the fishes and might be able to reduce the high blood pressure. In the study, exposure of lumpsucker to warm acclimation stresses 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 kept above 80 % throughout the experiment acclimation period and hypoxia-induced pathways might be supressed by cold acclimated stress, however, it remains unclear whether fishes were hypoxic or not and where they can develop a resistance to hypoxia. APO-E was upregulated in short-term warm acclimated fishes may be due to lipid membrane alteration in the skin. The immune response proteins ANXA3, MFAP and APO-E were expressed highly and may indicate immune stimulation in the temperature stressed fish. Pre-acclimation of fishes to thermal stress can activate the acclimation pathways and increase the tolerance to thermal stress. However, one of the goal of this study was to gain a broad understanding of heat response in lumpsucker in the varied temperature and to provide early insights into the important processes and pathways that may be helpful for the further studies.

5.2. Isolated mannose binding proteins in lumpsucker skin:

In the present study, mannose sepharose was used to identify the mannose binding proteins in the skin. Mannose binding lectins were well known for the pathogen recognition, activate complement system. Isolation of MBL by affinity chromatography has been used in *Petromyzon marinus; Ictalurus furcatus* (Ourth et al., 2007), *Cyprinus carpio* (Nakao et al., 2006), *Clarias gariepinus*

(Argayosa et al., 2011). In the present study, mannose sepharose used to separate the mannose binding protein and total five proteins were identified might be due to proteins carbohydrate specificity such as mannose, glucose, L-fucose, N-acetyl-mannosamine (ManNAc), and N-acetyl-glucosamine (GlcNAc) which are binding to mannose sepharose column. The binding of proteins other than lectin could also be due to their high affinity to high mannose N-glycan. However, it cannot be exclude that some of the isolated proteins were interacting with mannose binding proteins and due to this were indirectly bound to the column The importance of glycans in the immune system has been extensively described, with major roles in cell to cell adhesion and as recognition signals for antibodies (Rudd et al., 2001). Further, size-exclusion gel filtration was used to separate the protein according to fraction it was not successful, but the third band from the mannose sepharose affinity (Fig. 3a) was separated successfully into two bands (bands in Fig. 3a and Fig. 3b) in in size-exclusion (sephacryl S 200 HR) column (Fig. 3b). Binding of isolated proteins happened in the presence of ions in the binding buffer such as Ca^{++} or Mg^{++} and these metals were necessary for the mannose sepharose column. In a previous study, mannan- agarose affinity column was used to purify mannose binding protein in the milk fish serum and also here a mixture of proteins bound to mannose was observed (Argayosa et al., 2014) and bands found had molecular weights of 55 kDa, 30 kDa and 12 kDa under reducing condition (using β-mercaptoethanol). The proteins present in the bands were not identified. In lumpsucker, under reducing condition five bands were observed after mannose sepharose and after size exclusion totally six bands were observed.

Of the six bands observed in our study, the band 1 in the gel, is not showing any significant hit in the MASCOT, it has a molecular weight at 76 kDa. There rest of the bands were identified as shown in Table. 5

Transferrin (TF): Glycoprotein with iron-chelating properties that transports and scavenges extracellular iron. Transferrin reduce the infection by binding iron making it unavailable for the microorganism. The TF contains two domains N-terminal half domain (N-lobe) and the C-terminal half domain (C-lobe), among two C-lobe may be primary binding site in the transferrin receptor (Zak and Aisen, 2002). Binding to the column also due to TF found to interact with mannose binding lectin (Childs et al., 1990). Serum derived proteins were also identified in the proteomic study in the mucus of the teleost (Easy and Ross, 2009, Provan et al., 2013). The TF is involving in macrophage activation pathway and may involve toll like receptors (TLR). The TLR have received considerable attention as innate pattern recognition receptors that are not only important in the recognition of pathogen associated molecular patterns (PAMPs), but are important in

initiation and transduction of the intracellular signals that induce innate immune mechanisms in macrophages (Medzhitov, 2001).

Glial fibrillary acidic protein (GFAP): In the cytoplasm, GFAP is a glial intermediate filament (IF) protein, it's role is well defined in human where it is expressed in the central nervous system but not much studied in skin of fish. The protein is known to interact with mannose binding lectins. It has been shown that GFAP binding to concanavalin A is inhibited by α-methyl D-mannopyranoside (Owen et al., 2009). In the fish, the exact role of GFAP has to be determined. They have been observed in three spots in a previous study on lumpsucker skin mucus (Patel and Brinchmann, 2017).

a1-antitrypsin: A serum glycoprotein**,** with serine protease inhibitor activity. The antitrypsin were observed in the skin mucus of stressed gilthead seabream (Pérez-Sánchez et al., 2017) and act as acute phase signal. The antitrypsin also has a role in controlling proteases in inflammatory, complement, and coagulation pathways (Gooptu and Lomas, 2009). Interestingly anti-trypsin has been shown to increase macrophages efferocytosis via mannose binding lectin-mannose receptor signalling suggesting that antitrypsin can bind mannose binding proteins (Sandhaus et al., 2017).

Fucose binding lectin: the identified fucose binding lectin has been detected in tissues and fluids of vertebrate and invertebrate species (Odom and Vasta, 2005). In the binding study, the fucose was isolated due to the N-terminal amino acid sequence which is sugar binding in the presence of calcium. Fucose binding lectins was earlier purified in the serum of several teleost fishes; Japanese eel *Anguilla japonica* (Honda et al., 2000) sea bass (*Dicentrarchus labrax*) (Cammarata et al., 2001), European eel (*Anguilla. anguilla*) (Bianchet et al., 2002), the striped bass *Morone saxatilis* (Odom and Vasta, 2005) and using fucose-agarose F-type lectin was purified in the sea bass and were independent of Ca^{++} (Salerno et al., 2009). Recently it was observed in the skin mucus proteome of European seabass (Cordero et al., 2015). Also it has been identified in bighead carp (*Aristichthys nobilis*) in gill and had an apparent molecular weight of 37 kDa (Pan et al., 2010) and are Ca^{++} independent. In the present study, the homologue hit was started to be 34 kDa in molecular mass in MASCOT (Table. 4) and band in our SDS was showing a 37 kDa band (fig. 3a and 3b). (Argayosa and Lee, 2009) observed fucose lectin in serum of Nile tilapia (*Oreochromis niloticus* L.*)*.

Pentraxin fusion protein- like: Pentraxin are evolutionary conserved protein component of extracelluar matrix, has specific calcium dependent ligand binding properties. Interestingly pentaxins have been found to form heterocomplexes with mannose binding lectin (Sandhaus et al., 2017). Two proteins recognised as pentraxin are serum amyloid P (SAP) and C-reactive protein

(CRP). In this study, mannose column isolated pentraxin in the SDS-PAGE was around 24 kDa and studies also revealed pentraxins are composed of multiple subunits with size varying between 20–30 kDa (Bottazzi et al., 2009). In a previous study, of the skin mucus of lumpsucker, pentraxin was observed (Patel and Brinchmann, 2017) and this study it was pentraxin fusion protein like in the skin of lumpsucker.

Of our five identified proteins isolated by the mannose column, only the fucose binding lectin is known to bind carbohydrate. Transferrin, glial fibrillary acidic protein, a1-antitrypsin, pentraxin fusion protein- like is all suggested to interact with mannose binding lectins. The fact that these proteins is isolated in the column thus could be an indirect binding where the fucose binding lectin bind mannose and the other proteins are co-isolated. Pentraxin could bind carbohydrates on some yeast in a galactomannan inhabitable manner, however this binding was not inhibited by mannose (Garlanda et al., 2002).

In this study, it is been chosen to use the term "mannose binding proteins" for simplicity since direct mannose binding cannot be excluded until further studies have been done. One band was not identified; in homology based proteomics it is quite common that proteins cannot be identified due to incomplete databases.

5.3. Hemagglutination and bacterial agglutination of isolated mannose binding proteins:

In this study, hemagglutination was observed in the isolated "mannose binding proteins" through mannose sepharose column. The isolated protein was showed hemagglutination in the highest concentration of 1 mg/ml, in the presence of Ca^{++} (10 mM) and in the absence, hemagglutination was not observed in the trypsin treated and glutaraldehyde fixed horse erythrocytes (Fig. 5). EDTA (10 mM) was used to inhibit the isolated proteins to chelate out Ca^{++} and inhibition was observed. In the holothurian (*Apostichopus japonicas*) coelomic fluid, a mannose binding lectin MBL-AJ showed a maximum hemagglutination activity to human erythrocyte in the range 10–40 mM (Bulgakov et al., 2007). In the sea lamprey plasma, mannose binding C-type lectin was also shown to be calcium-dependent after being eluted in a mannan–agarose affinity column with EDTA (Ourth et al., 2008). In our study, hemagglutination was successful for isolated proteins dialysed for 12 h in EDTA then in Ca^{++} for 12 h at 4 ℃ (Fig. 6). Other study showed, in Indian catfish the activity of the serum lectin was also found to be Ca^{++} dependent as evidenced by a complete loss of activity in the presence of EDTA; the loss of Ca^{++} by calcium chelators could cause irreversible damage to the active sites of lectin (Manihar and Das, 1990).

In the present lumpsucker study EDTA did not damage the active site as addition of Ca^{++} gave hemagglutination. In our study, two buffers have been used, TBS binding buffer and PBS

(Phosphate buffered saline). PBS was used initially, to check the effect of hemagglutination, problem with the PBS was Ca^{++} in the PBS precipitates and it may alter or inhibit the hemagglutination activity. In the hemagglutination, to check the titre of isolated proteins, 1 mg/ml concentrated proteins were serially diluted in the next step in presence of erythrocyte and TBS-I buffer and none of the diluted wells in microplate showed hemagglutination activity. Showing that isolated proteins required higher amount of proteins to hemagglutinate.

Agglutination:

Agglutination is an important part of the innate immune responses, it is mediated by specific proteins that recognize microbe-specific pathogen-associated molecular pattern molecules, LPS (lipopolysaccharide) of Gram negative bacteria, lipoteichoic acid of Gram positive bacteria, mannan and beta glucans of yeast and fungal cell walls (Mirelman et al., 1980). In the present study, *A. salmonicida*, a Gram-negative bacterium was used and the results slightly differ from hemagglutination, in all the isolated tests agglutination was observed in the presence and absence of Ca^{++}, this could be because multiple proteins were present. Perhaps some proteins are involved in agglutination and others in hemagglutination, or maybe one protein is using different functional groups for hemagglutination and agglutination. It may also be that the proteins were not effectively dialysed in 3 k spin filter and some Ca^{++} was remaining. Interestingly, in the presence of Ca^{++}, agglutination was stronger (Fig. 7(e)). Lectins earlier identified in rainbow trout serum by Ca^{++} dependent binding to *A. salmonicida* lipopolysaccharide (Hoover et al., 1998) and in the serum of the sea lamprey C-type lectin had specificity for mannose and in the *A. salmonicida* demonstrated that the lamprey MBL lectin specifically bound to mannose on the bacterial surface (Ourth et al., 2008). In our study, it the protein mixture showed agglutination among them some might have specificity to mannose for bacteria. In the catfish, intelectin showed agglutination activity against the pathogenic bacterium *A. salmonicida*, suggesting that the lectin plays an important role in self-defence against bacteria in the skin surface of the catfish (Tsutsui et al., 2011).

In the both hemagglutination and agglutination mucus was successful due to its antibacterial properties, were pathogens might be immobilised before they reach to the epithelial cells and mucus known to be rich in humoral innate immune factors. The mucin molecule in the mucus make bacteria to adhere. Mucin is highly glycosylated glycoproteins and research also found antimicrobial properties in the fish against pathogens in epidermal mucus (Ángeles Esteban, 2012) and in our study crude mucus and isolated mucus proteins (gel result in Appendix IV, similar bands were detected but not characterised) were used from lumpsucker (Fig. 1) to check the agglutination (Fig. 5) and hemagglutination (Fig. 4a and 4b). The activities were successful and further studies

could be done to isolate the pure mannose binding proteins from the larger lumpsucker or numerous lumpsuckers to get more material.

The mannose-binding lectin has been proposed as a biomarker for disease resistance in vertebrates (Ourth et al., 2007). In the isolation of mannose proteins from skin of lumpsucker, we found important proteins which are known to be welfare biomarker such as antitrypsin, transferrin which were also observed in plasma for the physiological stress (Bohne-Kjersem et al., 2009). Identified proteins other than lectin may involve in pattern recognition based upon the PAMPs or DAMPs (danger-associated molecular patterns) and may be involved in innate mechanisms.

5.4. Methodology:

In this study, the sample was prepared by pulverising skin tissues in liquid nitrogen and in the presence of protein inhibitors in the binding buffer. Later, sonicated twice on ice to avoid over heating of sample. Initial in the isolation method samples were prepared without sonication and the amount of protein at end was less comparatively with sonication. Based upon the previous study, differentially expressed immune relevant genes in the dorsal and ventral part of the Atlantic cod skin (Caipang et al., 2011), samples were taken for the acclimation experiment in the lateral region, in between gill arch and dorsal fin. In the mannose isolation process, column was equilibrated and two hours used to bind the proteins other studies also suggest overnight binding. In the catfish, for the isolation of intelectin, mannose agarose column was left to bound sixteen hours (Tsutsui et al., 2011) and in milk fish mannose agarose for one hour incubation to isolate mannose bound proteins from the serum (Argayosa et al., 2014). During isolation in the study, proteins were eluted using 0.5 M D-mannose to isolate mannose binding proteins and some studies also suggest using methyl-α-D-mannopyranoside is an effective method to purify and isolate mannose-binding proteins from teleost sera (Argayosa et al., 2014). Further studies are needed to purify Ca^{++} binding proteins or identified fucose lectin. A purified protein will make it possible to make further functional studies. Purification of proteins could be achieved by other gel filtration column or the by changing the buffer in the same sephacryl S 200 HR tried already. The sepharose and sephadex have been widely used absorbents to isolate proteins. In the previous study, Sephacryl S-200 gel filtration was used for purification of galectin-1 from Atlantic cod skin mucus (Rajan et al., 2013b) and in bighead carp (*Aristichthys nobilis*) a novel fucose binding lectin from the gill tissues was isolated by DEAE-Sephrose FF followed by gel filtration sephacryl S 200 HR and Superdex 200 10/300 GL (Pan et al., 2010). In the present study, only gel filtration by sephacryl S 200 HR was used, unsucessful may be beacause of the buffer.

In the study, gel based technique SDS-PAGE was used to separate the proteins on molecular weight under reducing condition. Some of the challenges observed in the gel based techniques are gel-to-gel variation, limited linear dynamic range (LDR), limited throughput, advantages are ability to observe even small post-translational changes and highly homologous isoforms can often obtain directly. Gel free method was also important with deeper proteomic coverage but in this study, gel based method was used since this is the most convenient way to identify proteins by homology matching important when limited molecular data is available as is the case with lumpsucker. For the isolation study, one dimensional and for acclimation work two-dimensional electrophoretic method were used. 2DE was applied in the acclimation work to separate the complex mixture of proteins by segregating according to p*I* first dimensional gel and size in the second dimension SDS-PAGE. Protein extraction and solubilization are the key steps for proteomic analysis and mainly depend on the solubilization buffer used in the IEF step (Magdeldin et al., 2014). Proteins in their extremely acidic and basic were missing in the study due to use of IPG strips with a range of 3-10 pH and are unable to segregate in 2DE. However, this is a minor problem since most biological proteins are in the 3-10 range. In the study, Colloidal Coomassie and Sypro® Ruby stains were used to visualise the proteins in the gels, Colloidal Coomassie G-250 was used in 1DE and are less sensitive than the Sypro® Ruby. Coomassie stain was not used for 2DE due to Sypro® Ruby staining has been reported to yield a greater limited linear dynamic range, and reduced interprotein variability (IPV) compared with Colloidal Coomassie blue (CCB) and silver stains (Lanne and Panfilov, 2005, Tannu et al., 2006). Spots obtained in the 2DE was further analysed by PDQuest software (Bio-Rad) automated methods are helpful for gel identification particularly in the quantitative proteomics, in the study, missed spot gave zero value in the data inorder to make reliable, imputing values for missing spot intensities by manually in the gels using PDQuest software to normalise and validate the data statistically. To check the normality of the data, GraphPad Prism 7 used. We used manual method and spots may be disappeared in the treated group gels, disadvantage of software is it does not showed any value for missing spot, similar zero value in PDQuest observed in the database of spot intensities and then normalised to validate statistical protocol for 2DE (Meleth et al., 2005). The protein spots from the gels were then incubated with the enzyme trypsin to digest the protein to peptides. Before the application of mass spectrometry, the resulting peptides were extracted from the gel pieces and the peptide mixture analysed by ESI-Quad-TOF. Spectra were searched against the Actinopterygii in search databases NCBI and SWISprot in MASCOT software which is freely accessible database. although so far there is no genome data available for the lumpsucker. By using the peptides, homology searches against previously identified sequence in other fishes are performed. Studies

also suggest that the lack of species-specific databases for protein identification in marine species is by far the most significant limiting factor in proteomics research (Slattery et al., 2012). Once the lumpsucker genome will be available in database, it will provide very relevant information for future proteomic studies.

6. Conclusions:

This study shows preliminary results of the analysis of isolated mannose binding proteins and characterization of differentially expressed proteins in lumpsucker after temperature change. Hemagglutination and microbial recognition are some of the properties of mannose binding proteins in innate immunity. This study reported the agglutination activity of lumpsucker mannose binding proteins against *A. salmonicida* and hemagglutination. Proteomics is a powerful approach to assess the proteins involved in adaptation to a changing temperature, where organism has to shift its thermal optimum for numerous physiological activities to new higher temperature ranges**.** Mass spectrometry-based proteomic approach identified proteins in the skin of lumpsucker after homology matching in public protein databases. Welfare biomarker discovery and validation is still a big challenge, for the new cleaner fish in aquaculture.

7. Further studies:

- To separate the proteins isolated by the mannose binding column from each other further work is need.
- The identified novel fucose binding lectin could be isolated by using fucose agarose affinity to characterise eg. its independent or dependent nature of cations.
- Based upon skin mucus results in hemagglutination and agglutination, mucus could be used to isolate mannose binding proteins from larger lumpsuckers.
- In a new acclimation trial, fishes could be back acclimatised to its original temperature and the proteome of this fishes checked against fish from the original temperature, 8 °C and 14 °C
- In a new temperature acclimation trial, sampling could be performed daily.

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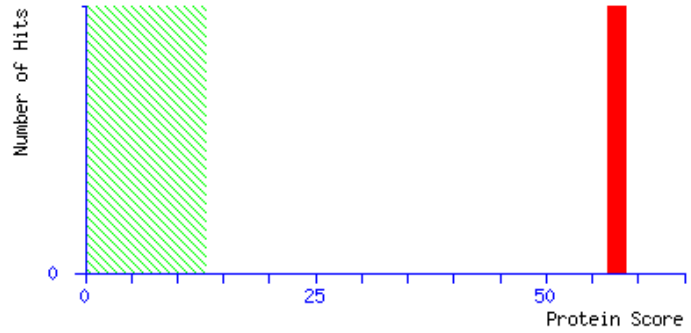
Appendix I

MASCOT results for identified spots in 2D analysis:

Spot 1:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

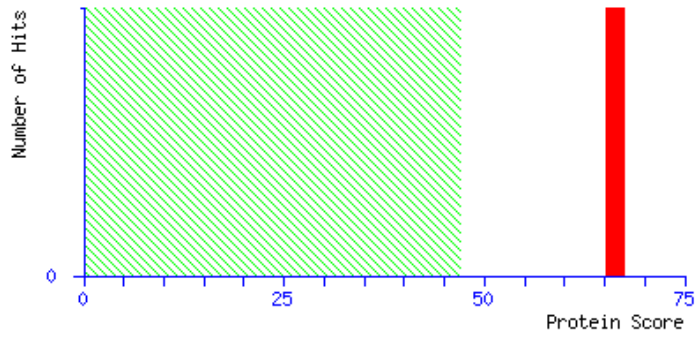


1. XP_010770112.1 Mass: 10885 Score: 58 Matches: 1(1) Sequences: 1(1)
PREDICTED: small ubiquitin-related modifier 2 [Notothenia coriiceps]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2	624.8211	1247.6276	1247.6510	-18.73	0	61		1	U	K.VAGQDGSIVQFK.I

Spot 3:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 47 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

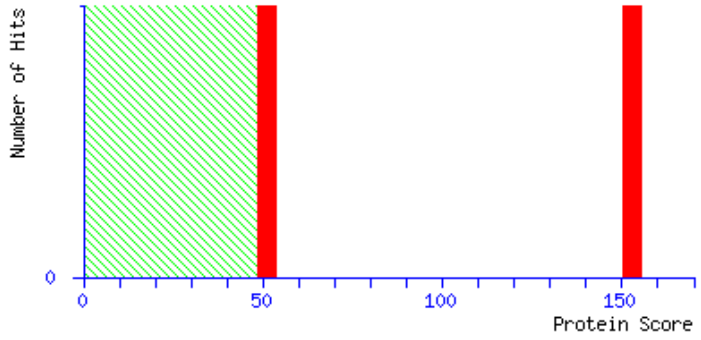


1. 1::KKF32533.1 Mass: 14604 Score: 66 Matches: 1(1) Sequences: 1(1)
Prefoldin subunit 5 [Larimichthys crocea]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
63	684.0126	2049.0158	2049.0630	-23.02	0	66	0.0007	1	U	K.TQLDQEIEFLTSSIGQLK.V

Spot 5:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

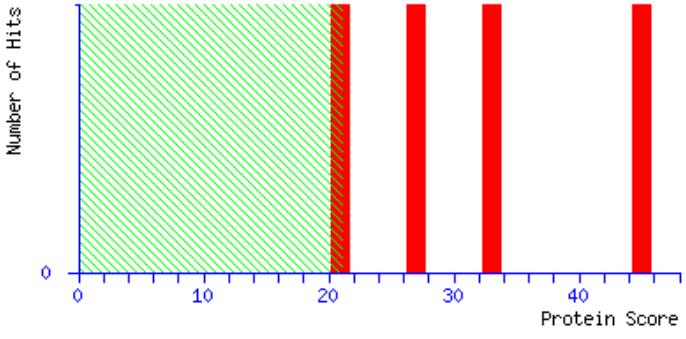


1. 1::ACO10145.1 Mass: 13128 Score: 153 Matches: 2(2) Sequences: 2(2)
SH3 domain-binding glutamic acid-rich-like protein [Osmerus mordax]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
28	650.3389	1298.6632	1298.6718	-6.57	0	89	4.9e-06	1	U	K.VYIASSSGSTSIK.K
43	756.8165	1511.6185	1511.6140	3.01	0	63	0.0011	1	U	K.YCGNYEAFFDAR.E

Spot 6:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 21 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

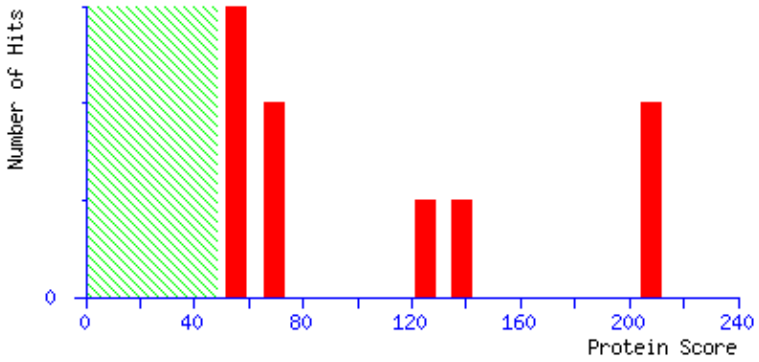


1. MGN_DANRE Mass: 17269 Score: 45 Matches: 1(1) Sequences: 1(1) emPAI: 0.35
Protein mago nashi homolog OS=Danio rerio GN=magoh PE=2 SV=1
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
47	587.3157	1172.6168	1172.6401	-19.84	0	45	0.00028	1	U	K.IGSLIDVNQSK.D

Spot 8:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

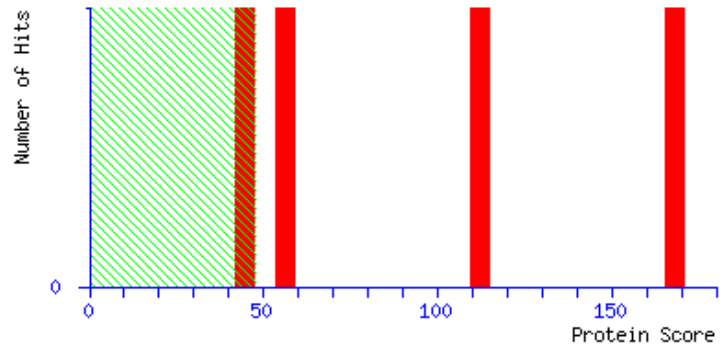


1. 1::AC009437.1 Mass: 26440 Score: 208 Matches: 4(4) Sequences: 3(3) emPAI: 0.81
Proteasome subunit beta type 1-A [Osmerus mordax]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
15	505.2308	1008.4471	1008.4876	-40.20	0	52	0.026	1		R.DVYTGDALR.I
36	574.7929	1147.5713	1147.5622	7.93	0	80	4.4e-05	1		R.LSEGYSIHSR.D
79	944.9669	1887.9191	1887.9434	-12.85	0	(62)	0.002	1	U	K.TMTSGAIAAMLSTILYGR.R + 2 Oxidation (M)
80	630.3168	1887.9287	1887.9434	-7.79	0	76	9.3e-05	1	U	K.TMTSGAIAAMLSTILYGR.R + 2 Oxidation (M)

Spot 9:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

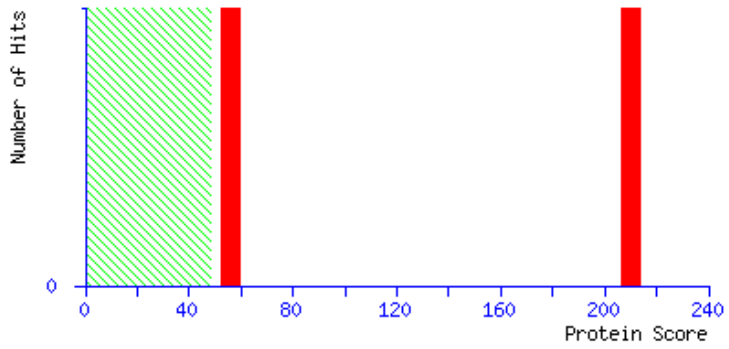


1. 1::AAI54779.1 Mass: 48095 Score: 168 Matches: 2(2) Sequences: 2(2)
Retinoblastoma binding protein 4, like [Danio rerio]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
40	600.2790	1198.5435	1198.5619	-15.32	0	105	1.2e-07	1	U	K.GAEFGGFGSVSGK.I
70	743.3711	1484.7276	1484.7399	-8.31	0	63	0.002	1	U	K.TPTSDVLVFDYTK.H

Spot 10:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

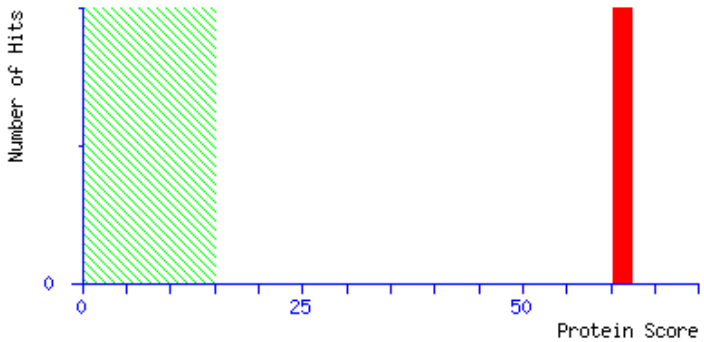


1. 1::XP_006783765.1 Mass: 52208 Score: 210 Matches: 3(3) Sequences: 3(3)
PREDICTED: angiotensinogen-like [Neolamprologus brichardi]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
51	728.8581	1455.7017	1455.7569	-37.96	0	91	2.8e-06	1	U	K.TLQGINSLVDDGPK.D
52	733.8515	1465.6884	1465.7202	-21.65	0	57	0.0081	1	U	K.DEITTQVWAFTR.Q
91	968.8012	2903.3819	2903.4665	-29.14	1	62	0.0011	1	U	K.TLQGINSLVDDGPKDEITTQVWAFTR.Q

Spot 11:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

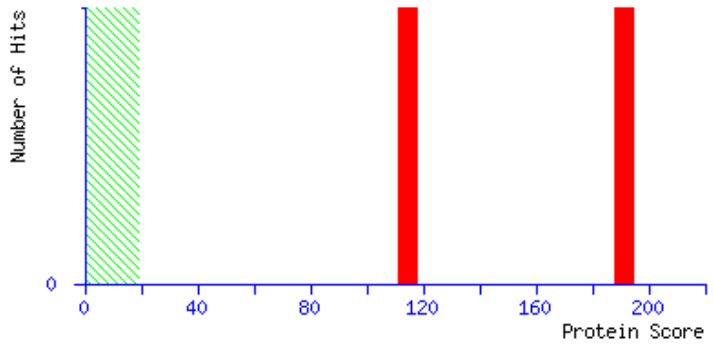


1. 1::XP_018936831.1 Mass: 28240 Score: 61 Matches: 1(1) Sequences: 1(1)
PREDICTED: microfibril-associated glycoprotein 4-like [Cyprinus carpio]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
25	580.3018	1158.5891	1158.6146	-21.95	1	65		1	U	K.DKGGWTVIQR.R

Spot 12:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

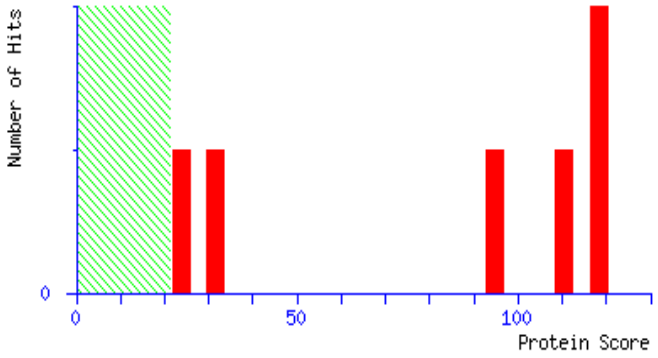


1. 1::ACF21982.1 Mass: 31040 Score: 191 Matches: 5(5) Sequences: 4(4)
apolipoprotein E [Oplegnathus fasciatus]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
20	520.2677	1038.5208	1038.5233	-2.39	0	20		1		R.STEYLGELK.T
59	726.3754	1450.7363	1450.7416	-3.64	0	85		1		R.NTVATYLGELQSR.T
60	484.5874	1450.7404	1450.7416	-0.82	0	(56)		1		R.NTVATYLGELQSR.T
73	625.6482	1873.9227	1874.0149	-49.21	0	35		1	U	K.IDELTELLSPYATQIR.E + [+13.0316 at T13]
75	629.6486	1885.9239	1885.9381	-7.52	0	67		1	U	R.TQAEGLGQQLEIQAEGLK.T + [-14.0157 at T12]

Spot 14:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 21 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



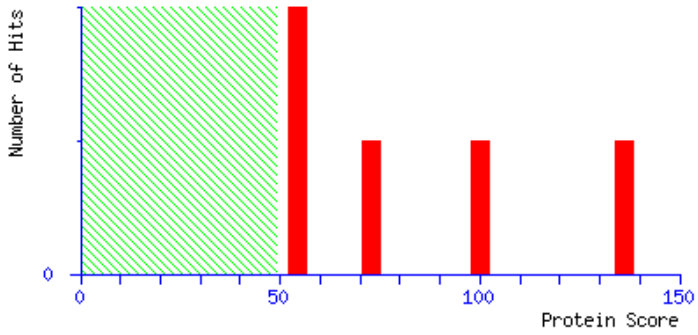
1. RSSA_SALSA Mass: 35043 Score: 118 Matches: 5(5) Sequences: 5(5)
40S ribosomal protein SA OS=Salmo salar GN=rpsa PE=2 SV=1

Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
10	463.7798	925.5451	925.5597	-15.77	0	30	0.0048	1	U	R.LLIVTDPR.A
54	653.8288	1305.6431	1305.6387	3.36	0	29	0.0088	1		R.YVDIAIPCNNK.G
60	441.8754	1322.6044	1322.6368	-24.45	0	62	5.1e-06	1		K.FASATGATTFHGR.F
83	849.9079	1697.8012	1697.8526	-30.25	0	43	0.00037	1		R.FTPGTFTNQIQAAFR.E
85	907.4548	1812.8950	1812.9404	-25.04	0	28	0.0088	1		R.AIVAIENPADVCVISSR.N

Spot 15:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 49 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

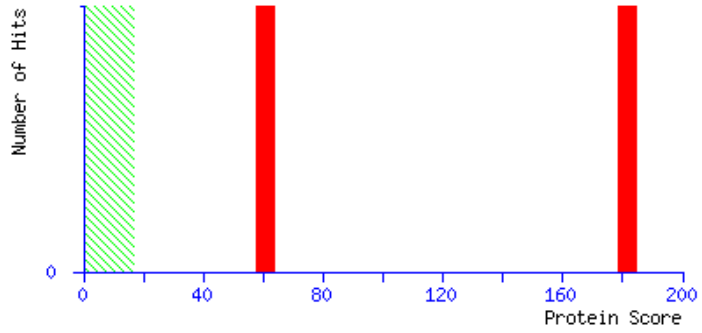


1. KKF17944.1 Mass: 36677 Score: 136 Matches: 2(2) Sequences: 2(2)
Annexin A3, partial [Larimichthys crocea]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
17	536.8331	1071.6516	1071.6288	21.3	0	72	0.00021	1	U	K.TLIEVLTQR.S
18	537.3042	1072.5939	1072.5764	16.3	0	66	0.0014	1	U	R.SEIDLLDIR.A

Spot 16:

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. XP_010780849.1 Mass: 27155 Score: 181 Matches: 6(6) Sequences: 3(3)
PREDICTED: catechol O-methyltransferase domain-containing protein 1-like [Notothenia coriiceps]
Check to include this hit in error tolerant search

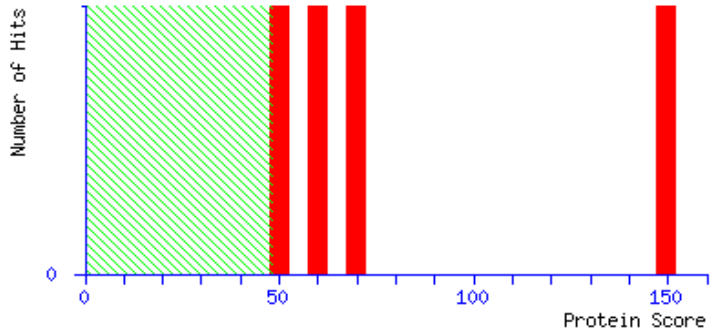
Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
52	772.4058	1542.7970	1542.8406	-28.24	0	97		1	U	R.TGGIIAIDNVLWSGK.V
55	780.4252	1558.8359	1558.8355	0.23	0	(29)		1	U	R.TGGIIAIDNVLWSGK.V + [+15.9949 at W12]
56	794.4244	1586.8343	1586.8304	2.45	0	(27)		1	U	R.TGGIIAIDNVLWSGK.V + [+43.9898 at W12]
67	665.6860	1994.0363	1994.0684	-16.10	0	(22)		1	U	K.VVNPAPSDLTSQALDALNK.K + [+42.0470 at D8]
69	708.3505	2122.0298	2122.1270	-45.78	1	43		4	U	K.VVNPAPSDLTSQALDALNKK.L + [+42.0106 at S7]
70	708.3820	2122.1242	2122.0347	42.2	0	52		1	U	K.VVNPAPSDLTSQALDALNK.K + [+170.0133 at S7]

Appendix II

Mascot results for isolated protein from mannose sepharose column:

2.

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



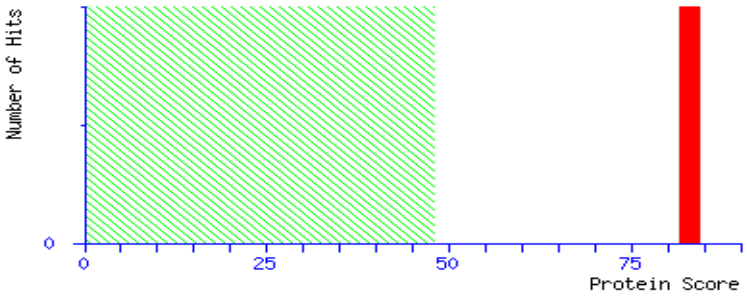
1. 1::AEV21971.1 Mass: 75092 Score: 149 Matches: 2(2) Sequences: 2(2) emPAI: 0.15
transferrin [Trachidermus fasciatus]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
21	520.2784	1038.5422	1038.5346	7.40	0	58	0.0066	1	U	K.HLTVPESEK.A
101	849.8531	1697.6915	1697.7567	-38.36	0	91	1.9e-06	1	U	K.EADAMAVDGGQVYTAGK.C + Oxidation (M)

Proteins matching the same set of peptides:
1::ALP46207.1 Mass: 76080 Score: 149 Matches: 2(2) Sequences: 2(2)
transferrin [Lutjanus argentiventris]
1::AAP94279.1 Mass: 76146 Score: 148 Matches: 2(2) Sequences: 2(2)
transferrin [Pagrus major]

3a.

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

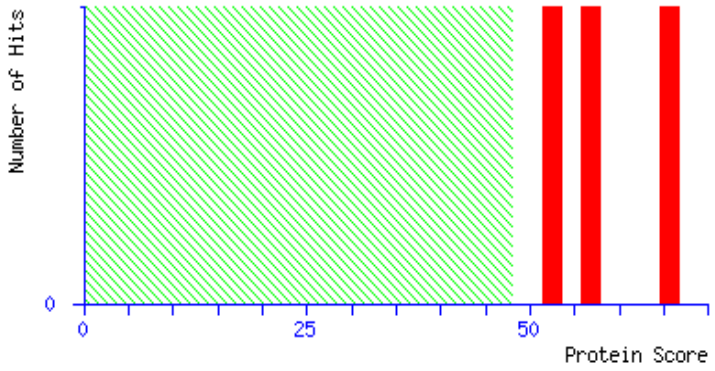


1. 2::GFAP_CARAU Mass: 42636 Score: 83 Matches: 1(1) Sequences: 1(1)
Glial fibrillary acidic protein (Fragment) OS=Carassius auratus GN=gfap PE=2 SV=2
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
37	639.3846	1276.7547	1276.7027	40.8	0	83	1.6e-05	1	U	K.LALDIEIATYR.K

3b.

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. 1::KPP69326.1 Mass: 55997 Score: 66 Matches: 1(1) Sequences: 1(1)
keratin, type II cytoskeletal 8-like, partial [Scleropages formosus]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
16	517.2834	1032.5522	1032.5087	42.1	0	66	0.0017	1	U	R.TLLEGEESR.L

2. 1::AMQ75915.1 Mass: 46723 Score: 55 Matches: 1(1) Sequences: 1(1)
a1-antitrypsin [Monopterus albus]
Check to include this hit in error tolerant search

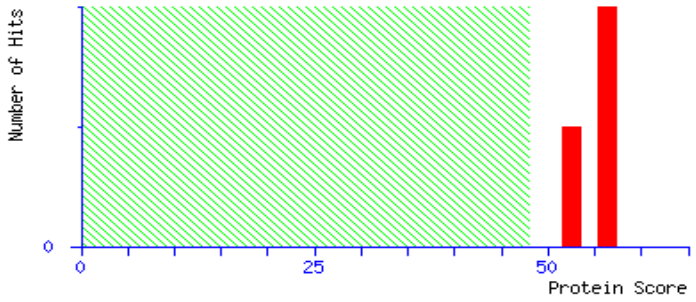
Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
53	764.4195	1526.8245	1526.7769	31.2	0	55	0.012	1	U	K.LSAPNADFAFALYK.S

Proteins matching the same set of peptides:

4.

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



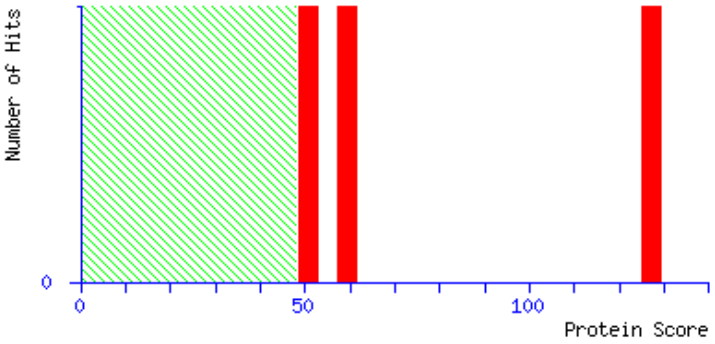
1. AGT40149.1 Mass: 34580 Score: 56 Matches: 1(1) Sequences: 1(1)
fucose-binding lectin [Trachidermus fasciatus]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1	480.2854	958.5562	958.5600	-3.91	0	56	0.012	1	U	R.YVNVVIPR.E

5

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. XP_016093745.1 Mass: 25478 Score: 127 Matches: 2(2) Sequences: 2(2)
PREDICTED: pentraxin fusion protein-like [Sinocyclocheilus grahami]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
13	501.7782	1001.5419	1001.5142	27.7	0	58	0.0085	1	U	R.VATELQGER.Q
40	696.3341	1390.6537	1390.6517	1.39	0	69	0.00059	1	U	R.TPDFDELNVWR.E

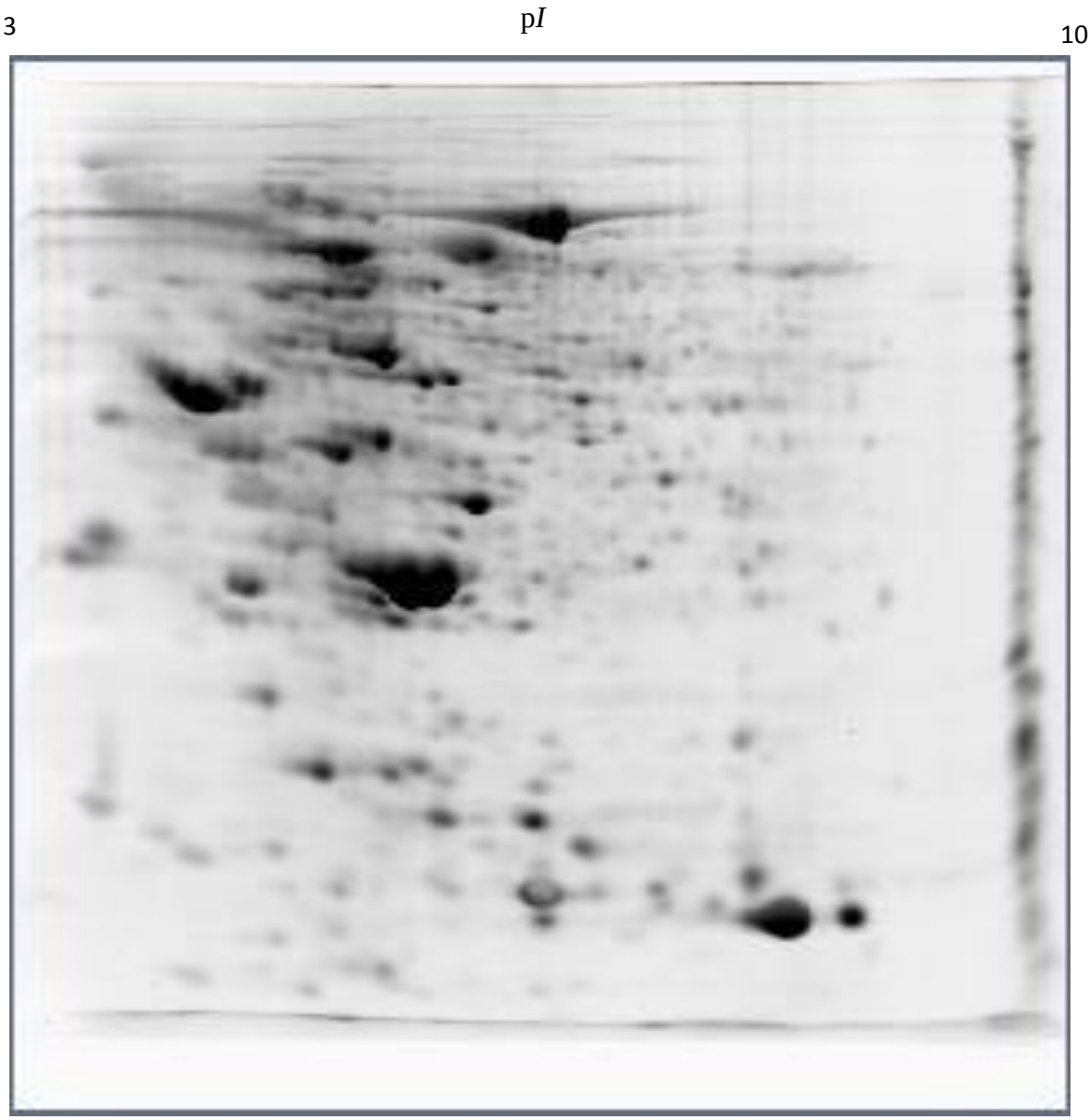
2. ADX06859.1 Mass: 25060 Score: 58 Matches: 1(1) Sequences: 1(1)
C-reactive protein [Lates calcarifer]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
13	501.7782	1001.5419	1001.5505	-8.64	1	58	0.0091	1	U	R.VATELKGER.E

Appendix III

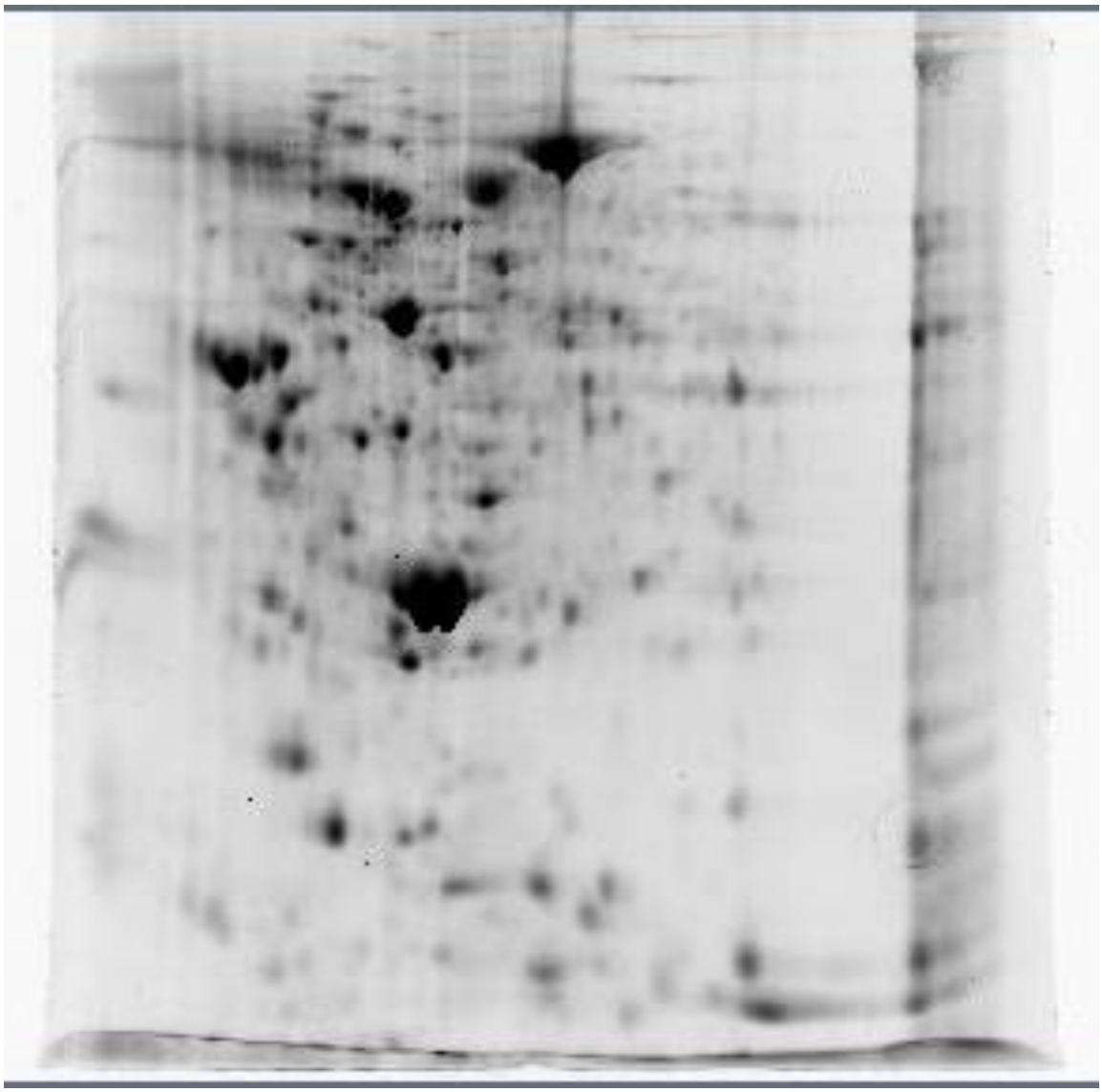
2DE gels used in temperature acclimation experiment

Cold acclimation (8 ℃) gels

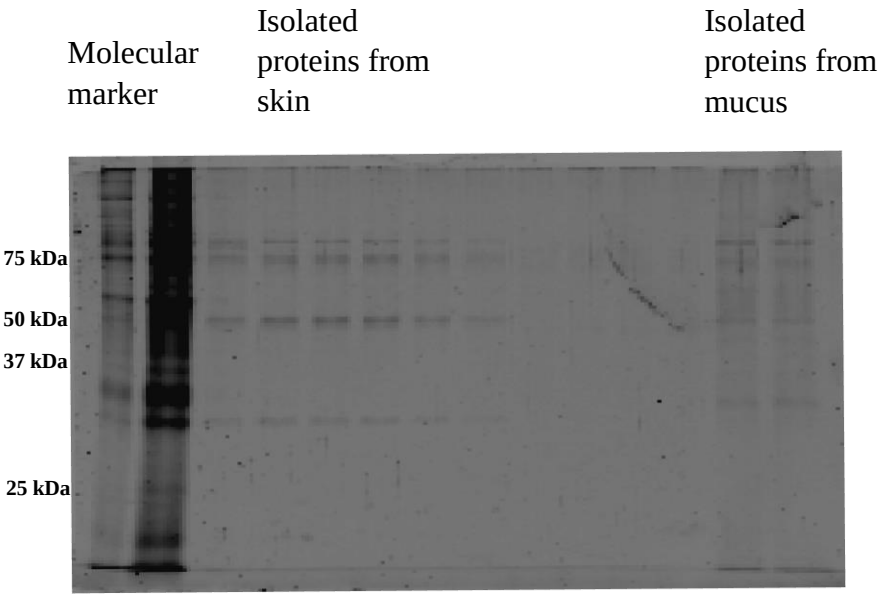


Warm acclimation (14 ℃) gels

3 p*I* 10



Appendix IV


Molecular marker
Isolated proteins from skin
Isolated proteins from mucus
75 kDa
50 kDa
37 kDa
25 kDa