Nutritional and environmental impacts on skin and mucus condition in Atlantic salmon (*Salmo salar* L.)

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Contents

Abstract

The skin and associated mucus layer of Atlantic salmon constitutes its first line of defence against the aqueous environment. Through intensive farming, a range of stressors including both mechanical and environmental factors are known to have an impact on the skin condition of fish. Damaged skin can serve as a portal of entry for primary pathogens and secondary infections. Two of the current main problems in the salmon farming industry are skin related: ectoparasitism with sea lice and skin wounds of diverse origin, often related to extreme temperatures.

Four experiments with salmon were conducted to investigate the response of skin and the epidermal mucus layer to challenges representative of normal farming conditions, and to assess the possibility to modulate the skin response through diet. The factors studied included water temperature change, sea lice infection and mechanical wound infliction. New methodologies were developed or existing methods optimised to assess the impact of these factors on the composition, structure and functionality of skin. Quantitative histology using digital image analysing, proteomics of mucus and transcriptomics of skin were all demonstrated to be valuable tools in defining differences between groups exposed to distinct treatments.

The chemical composition of the skin was studied at 3 temperatures; 4°C, 10°C and 16°C. Increasing temperature resulted in higher level of protein and collagen related amino acids, while moisture level was reduced. Altering the dietary levels of minerals and vitamins also resulted in changes in their concentration in the skin, demonstrating dietary modulation of skin composition. The structure of the epidermis was also affected by temperature. Quantitative histology assessments showed that the epidermal thickness decreased from low to high temperature, whereas the epidermal area comprising mucous cells increased. Temperature also impacted on the skin transcriptome. A subtle increase in skin-mediated immunity was observed at low temperature, suggesting a pre-activation of the skin mucosal immunity. Up-regulation of a number of heat shock proteins

correlating with a decrease in epidermal thickness, was consistent with a stress response in the skin of fish exposed to high temperature.

The effects of temperature (4°C and 12°C) and diet composition were studied on the healing process after infliction of mechanical wounds. During the healing process, the changes observed in the epidermis were affected by time, temperature and diet. Elevated temperature and additional dietary Zn accelerated wound healing, as assessed by the mucous cell distribution within the epidermis at two weeks post wounding. The expression of three newly-described salmon chemokines (CK 11A-C) was also found to be modulated in relation to skin damage. The positive effect of dietary Zn shown by histology was positively correlated with an increase in skin chemokine expression.

In two experimental trials, Atlantic salmon were fed diets containing functional ingredients and challenged with sea lice. Changes in mucus composition after lice infestation were assessed by proteomics. A large number of proteins (more than 500) were identified in the mucus, many of which were related to immune and stress responses, underlining the importance of skin mucus as a first line of defence in fish. Several of the proteins responded to both sea lice infection and dietary modulation (chemokines, HSPs, AG-2, calmodulin and peptidyl-prolyl cis-trans isomerase). After further validation, these proteins could potentially be used as biomarkers to assess skin functionality. No impact on epidermal thickness or mucous cell area was seen in the challenge trials, indicating that these two structural parameters alone cannot explain the susceptibility to lice attachment. Some of the diets tested did reduce the number of attached lice. Although the reduction was not drastic, it clearly showed the potential of functional diets to modulate lice attachment.

List of publications

Paper I

Linda B. Jensen, Sebastian Boltana, Alex Obach, Charles McGurk, Rune Waagbø and Simon MacKenzie. 2014. Investigating the underlying mechanisms of temperature related skin diseases in Atlantic salmon, *Salmo salar* L.: as measured by quantitative histology, skin transcriptomics and composition. Journal of Fish Diseases, doi:10.1111/jfd.12314.

Paper II

Linda B. Jensen, Thomas Wahli, Charles McGurk, Tommy Berger Eriksen, Alex Obach, Rune Waagbø, Ana Handler, Carolina Tafalla. 2015. Effect of temperature and diet on wound healing in Atlantic salmon (*Salmo salar* L.). Submitted to "Fish Physiology and Biochemistry".

Paper III

Fiona Provan, Linda B. Jensen, Kai Erik Uleberg, Eivind Larssen, Tarja Rajalahti, Julia Mullins and Alex Obach. 2013. Proteomic analysis of epidermal mucus from sea lice infected Atlantic salmon (*Salmo salar* L.). Journal of Fish Diseases, 36:311-321.

Paper IV

Linda B. Jensen, Fiona Provan, Eivind Larssen, James E. Bron and Alex Obach. 2014. Reducing sea lice (*Lepeophtheirus salmonis*) infestation of farmed Atlantic salmon *(Salmo salar* L.) through functional feeds. Aquaculture Nutrition, doi: 10.1111/anu.12222

These four papers are referred to in the text by their roman numbers. Reprints were made with permission from the Publisher.

1. Introduction

1.1 General introduction

The aquaculture industry continues to be one of the world's fastest growing food producing sectors. In 2012 the production provided close to 50% of all fish for human consumption, and the share is predicted to rise to 62% by 2030 (FAO 2014). The salmon share of production is also increasing, and there has been an expansion into new markets and production areas. Norway remains the largest producer and exporter of Atlantic salmon with 1 143 700 tons in 2013, but to secure a sustainable industry there are a number of fish health aspects that needs to be improved. Through intensive farming salmon are exposed to a range of stressors, including mechanical and environmental factors. These can all have impacts on the fish skin and mucus layer, and thereby compromise the salmon's first line of defence. Small skin wounds and scrapes are common during handling or under suboptimal environmental conditions, and can serve as a port of entry for pathogens and secondary infections. One of the largest problems for the industry today is the sea louse (*Lepeophtheirus salmonis*). This ectoparasite feeds on the mucus and skin of salmon, and can cause lesions, osmotic problems and also secondary infections (Grimnes and Jakobsen 1996, MacKinnon 1993, Nolan *et al.* 1999).

Diets with functional ingredients are becoming a part of the preventative health strategy in fish farms (Bricknell and Dalmo 2005, Covello 2012). A strengthening of the skin and mucus layer through dietary modulation could play a role in preventing damage, parasite attachment and also promote faster recovery of damaged skin.

1.2 Skin structure

The structure of fish skin is highly adapted to the aqueous environment. Two main features makes fish skin differ from other vertebrates. Firstly, it has no keratinized outer layer, so the viable and active epithelial cells are in direct contact with the environment. Secondly, in most species a large part of the body is covered with scales. The fish skin is a large organ that is continuous with the linings of all body openings, and also covers the fins. It is a multifunctional organ, and serves roles in protection, communication, sensory perception, locomotion, respiration, ion regulation, excretion and thermal regulation (Whitear 1986).

In general, the skin consists of an outer mucus layer with a complex composition (see 1.3 for description), the epidermis and the dermis (fig 1). The epidermis is a non-keratinized, squamous stratified epithelium that varies in thickness from approximately 3 to 20 cell layers (Ferguson 1989). The basic cellular element of fish epidermis is the epithelial cell (also called Malpighian, epidermal or filamentous cells). These cells are metabolically active and capable of mitotic division throughout all layers of the epidermis, although most common in deeper layers close to the basement membrane (Elliott 2000). Dead cells are regularly sloughed from the epidermal surface and replaced by living cells. The cells vary in shape depending on their position in the epidermis. The basal cells are cuboidal or columnar, whereas the superficial cells are often flattened. The superficial cells also exhibit microridges, which form fingerprint-like patterns, probably assisting in holding mucous secretions to the skin surface. These superficial epithelial cells are able to migrate fast, and rapidly cover wounds after mechanical damage (Bullock *et al.* 1978).

Tonofilaments form an important part of the cytoskeleton of individual cells, and the attachment of tonofibrils to the desmosomal plaques that join adjacent epithelial cells enables the epidermis to respond as whole to mechanical stress (Elliott 2000).

Salmon skin also contains mucous cells (goblet cells), unicellular glands common to most animal groups. Mucous cells are most frequently recognized in the middle to outer layer of epidermis (*stratum superficiale*). They differentiate from epithelial cells in the lower layer of the epidermis (*stratum spinosum*), and migrate towards the surface. Immature mucous cells are rounded, but become flattened laterally and develop vesicles which enlarge their size as they approach the outer layers (Harris and Hunt 1975). At the surface the mucous cells membrane ruptures at the apical point, the cell contents are then released and the cell is sloughed. Other fish species may have a variation of specialized cells in the epidermis, like sacciform cells, sensory cells, alarm cells, club cells or venom cells (Elliott 2000, Whitear 1986). These specialised cells are not present in salmon skin.

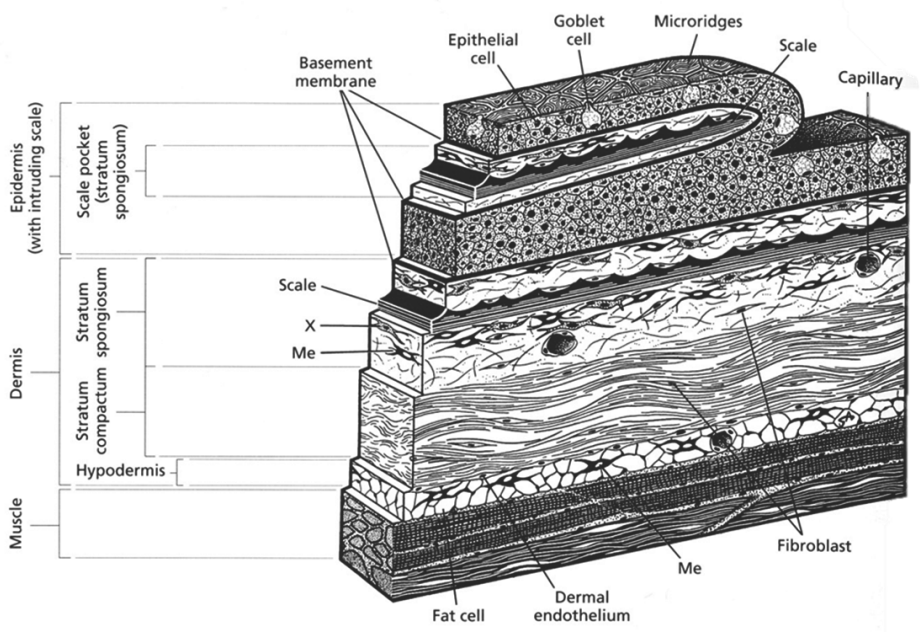

Figure 1. Three-dimensional section of salmonids skin, showing different skin layers and the major cell types of the epidermis and dermis. Abbreviations: *X* xanthophore; *Me* melanophore (Source: Elliott 2000).

The dermis is divided in two main layers. The outer, stratum spongiosum, is a loose network of connective tissue, containing fibroblasts, nerves and pigment cells. The inner layer, stratum compactum, is a denser layer consisting primarily of orthogonal collagen bands (Hawkes 1974). The stratum compactum is bounded by a single layer of cells named the dermal endothelium, separating it from a relatively well vascularized layer of loose connective tissue and adipocytes, the hypodermis. Below the hypodermis is the skeletal musculature.

Scales originate in scale-pockets in the dermis, and consist of a plate of collagenous tissue with superficial mineralization. Each scale is anchored in the pocket by bundles of collagen fibres, and the posterior end of the scale is projected into and covered by the epidermis.

The epidermis and the dermis are separated and anchored by a basement membrane that assists in controlling the passage of cells and molecules between the dermis and epidermis (Elliott 2000). It probably plays a role in wound healing, serving as an attachment site for epithelial cells.

1.3 Mucus

Fish skin mucus is a multifunctional material, and has functions in respiration, ionic and osmotic regulation, reproduction, communication, excretion and disease resistance (Alexander and Ingram 1992, Shephard 1994). The protective function of skin mucus is a combined result of mechanical and biochemical properties. The mucus is mainly secreted by the mucous cells in the epidermis, and acts as the first barrier to mechanical damage and infection. In addition to trapping and sloughing of pathogens, mucus contains a wide range of substances which can have effect on pathogens. The described components includes a number of innate immune components such as lectins, pentraxines, lysozymes, proteolytic enzymes, alkaline phosphatase, C-reactive protein, complement and

antimicrobial peptides as well as immunoglobulins (Alvarez-Pellitero 2008, Fast *et al.* 2002b, Guardiola *et al.* 2014, Jones 2001, Shephard 1994).

The main components of mucus are water and mucins. Mucins are high molecular weight, heavily glycosylated glycoproteins (>50% carbohydrates) which impart viscoelastic and rheological properties to the mucus. Mucins typically possess repetitive regions rich in threonine, serine and proline (Rose and Voynow 2006). In mammals the integrity of the mucus layer is often associated with nutritional state, for example, a limited dietary supply of threonine has been shown to impair the synthesis of mucins in rats (Faure *et al.* 2005). Not much is known about the impact of diet on mucus production in fish, but added dietary β-glucans did increase the expression of a mucine and two β-defencines in carp skin mucus (Marel *et al.* 2012). More than 20 human and murine mucin genes are described, but only two mucins have been cloned and sequenced in fish so far (Marel *et al.* 2012, Rose and Voynow 2006). In Atlantic salmon several isotigs with homology to mammalian mucins are found, but no full length mucins are described yet (Micallef *et al.* 2012).

The thickness of the mucus layer is proposed to be determined by the balance between the rate of secretion and rate of degradation and shedding. Toxic and irritating substances can stimulate mucus secretion and increase the thickness of the mucus layer (Esteban 2012), as can other stressors and pathogens (Fast *et al.* 2002a, Iger *et al.* 1995, Iger *et al.* 1994c). There is a variation in type and level of innate immune factors in mucus between species inhabiting different ecological niches, but also between different species of salmonids (Fast *et al.* 2002b, Mustafa 2005, Nigam *et al.* 2012, Subramanian *et al.* 2007). There is also evidence that the mucus composition varies with season, smoltification, salinity, stress, disease and parasite attack (Easy and Ross 2009, Easy and Ross 2010, Fagan *et al.* 2003, Guardiola *et al.* 2014, Lü *et al.* 2012, Mustafa 2005, Roberts and Powell 2005, Schrock *et al.* 2001).

Changes in mucus composition have been described in salmon infected with sea lice (Fast *et al.* 2002a, Jones 2001), and there are possibilities to utilize this knowledge for targeted immunological management (Fast 2014). Skin mucus can be a source for isolation of antimicrobial components that can be explored as treatments against pathogens, vaccine adjuvants or inactivated vaccines. More research is needed to get there, but in the meantime immunoprophylactic control by priming the mucosal barrier through diet could be useful.

1.4 Skin defence

Living in a pathogen-rich environment makes fish vulnerable to infections, and therefore reliant on a potent first defence line. Fish immune system is similar to that of higher vertebrates, but there are some differences (fig 2).

The immune system is commonly divided in two; the innate (non-specific) and the adaptive (specific) immune system. The innate immune response is an essential component in combating pathogens in fish, due to limitations of the adaptive immune system. It also plays a role in the adaptive response, through a system of receptor proteins. The innate immune system is commonly divided in three; the mucosal/epithelial barrier, the humoral parameters and the cellular components (Uribe *et al.* 2011). Skin mucus is secreted by cells in the epidermis, and acts as the first barrier to mechanical damage and infection. It contains several immune substances, and its function is described in chapter 1.3. The epidermis itself is also able to react to stressors resulting in a thickening or cellular hyperplasia (Uribe *et al.* 2011).

The humoral parameters are cellular receptors or molecules that are soluble in plasma or other body fluids (Magnadóttir 2006). Transferrin can act as a growth inhibitor of bacteria by chelating available iron essential for bacterial growth. It is also an acute phase protein invoked during inflammatory response, to remove iron from damaged tissue. Different proteases and protease inhibitors are also analysed in several marine fish

(Guardiola *et al.* 2014). Although their main functions are to maintain body fluid homeostasis, they are also involved in defence by obstructing pathogen invasion and viability (Ingram 1980, Uribe *et al.* 2011). Other immune-related enzymes are also active as defence elements, such as lysozyme, peroxidase and alkaline phosphatase (Ingram 1980, Magnadóttir 2006). Lysozyme possess lytic activity against Gram positive and Gram negative bacteria, and is known to activate the complement system and phagocytes (Saurabh and Sahoo 2008). Its activity has been described in a wide range of species, including salmonids (Fast *et al.* 2002b, Lie *et al.* 1989). The humoral parameters also include complement, pentraxines (C-reactive protein and serum amyloid protein), lectins and antimicrobial peptides (AMPs) (Alvarez-Pellitero 2008). Finally, cytokines and chemokines are important regulators of the immune system, and can have both pro-inflammatory, anti-inflammatory and pathogen-killing activities (Biswas *et al.* 2012). Chemokines are a family of small cytokines that regulate immune cell migration under both inflammatory and normal physiological conditions (Alejo and Tafalla 2011), and acts as a bridge between innate and adaptive response.

The main cellular components of the innate immune systems are leukocytes, including macrophages, neutrophils and lymphocytes. Phagocytosis is one of the main mechanisms involved in the clearance of pathogens, and mainly involves neutrophils, acidophilic granulocytes and monocyte-macrophages. The increase of phagocytic cells has been frequently reported in response to fish parasites (Alvarez-Pellitero 2008). Epithelial and dendritic-like cells also participate in the innate defence (Whyte 2007). Dendritic-like cells have recently been characterised in rainbow trout, and are described as specialized antigen presenting cells that bridge innate and adaptive immunity (Bassity and Clark 2012). Two types of natural killer (NK) cell homologues are also described in fish; non-specific cytotoxic cells and NK-like cells (Fischer *et al.* 2013).

Cell mediated immune response is complex and include components of both the innate (macrophages, NK cells etc.) and the adaptive (B- and T-cells) system. B-cells are involved in the humoral defence, while T-cells are responsible for cell-mediated

responses. Secretory immunoglobulins (Ig) produced by plasma cells are key players in maintenance of mucosal homeostasis. Teleost B cells produce IgM, IgD and IgT. IgM is the principle player in systemic immunity, while IgT appears to be specialized in mucosal immune response (Esteban 2012). Other lymphocytes can be divided into natural killer (NK) cells and T-cells. T-cells are again divided into cytotoxic lymphocytes (CTLs), T-helper cells and regulatory T-cells (Fischer *et al.* 2013).

The last few years the discovery of immune related genes and expression of the encoded protein has resulted in the establishment of specific antibodies against immune related proteins. The most important molecules in major immunological processes involving T cells are CD3, CD4 and CD8, as well as MHC class I and II (Fischer *et al.* 2013).

These innate and adaptive systems are shown to interact, where the innate response generally precedes the adaptive response, and activates and sets the nature of the adaptive response and thereby co-operates to maintain homeostasis (Magnadóttir 2006).

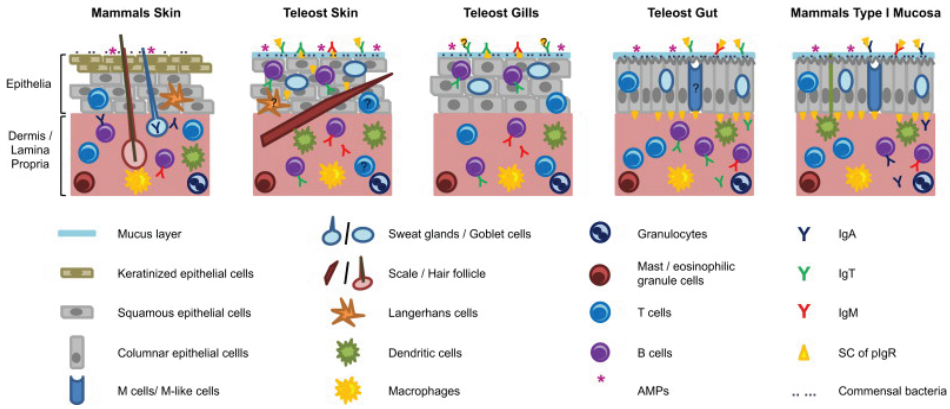

Figure 2. Schematic representation of similarities and differences between teleost fish skin, gills and gut, and mammalian skin and type I mucosal surfaces. Structural differences between the epithelia and dermis, and similarities in the cellular components of the innate immune system are displayed. Differences in the localization of B and T cells, the isotype of immunoglobulins and presence of the secretory component (SC) of the polymeric immunoglobulin receptor (pIgR) are represented as well. The presence of commensal bacteria and antimicrobial peptides (AMPs) is shown in the outer surface. Elements that are suspected to be present in a tissue, but have not been studied so far are marked as unknown (?).(Source: Gomez *et al*. 2013).

1.5 Impact of external factors on skin health

A range of external factors can have an impact on skin health under commercial farming conditions. The most important may be factors regarding water quality, e.g. water temperature, oxygen and salinity. In addition repeated handling of the fish through grading, transportation, net-changes, treatments and harvest may cause stress to the fish throughout the production cycle.

In general, stress in fish causes the release of innate immune factors into the serum to offset the effects of the stressor. There are many factors and their interactions that can act as stressors. Examples can be physical factors like exercise, pressure and silt, ecological factors which derive from social stress, predation and food availability, and chemical sources resulting from changes in pH, O_2, CO_2 and xenobiotics (Davis 2010). Several of these factors have been shown to have an impact on epidermal structure and mucous cell density in fish (Burkhardt-Holm *et al.* 1997, Christiansen *et al.* 1991, Iger *et al.* 1994a, Iger *et al.* 1994c, Iger and Wendelaar Bonga 1994, Kato *et al.* 2014). Whether the stressor is acute or chronic will also affect the impact on fish skin.

Temperature has a significant effect on poikilothermic animals like fish. All animals have their optimal temperature for growth and health, but are able to function within a wider temperature range. Atlantic salmon have a high temperature tolerance, and can survive in temperatures between 0-23°C (Priede 2002), with the optimal temperature for growth around 14°C (Handeland *et al.* 2008). The pathological situation in fish farming depends on temperature dependent regulation of the immune system, combined with the temperature specificity of the pathogens (Le Morvan *et al.* 1998). Most pathogens affecting farmed fish are temperature specific, and for salmon farmed in Norway it is clearly low temperature that causes most skin related problems. Winter ulcers affect fish mainly during the months from January to March (Lillehaug *et al.* 2003), when water temperatures are lowest. In the same period fish are vulnerable to handling and stress, and mechanical damage is well known to cause secondary infection at low temperatures. Low

temperatures affect both cellular and humoral immune responses in fish. The limit between immunologically permissive and non-permissive temperatures depends on the species, and is established at 4°C in salmonids (Bly and Clem 1992). Innate parameters are often still active at low temperatures, while adaptive parameters tend to be more suppressed. It seems like nonspecific defences tend to offset specific immune suppression at low temperature until the specific immune system adapts (Le Morvan *et al.* 1998).

1.6 Skin diseases

Farmed fish are affected by a variety of skin conditions, some of which have large impact on both economy and welfare. Skin ulcers can have a variety of aetiologies, including toxic agents, immunological causes, different infectious agents and nutritional deficiencies. Skin problems also include mechanical damage caused by netting, transport or other external stressors, and can lead to secondary infections. For many pathogens it is well established that skin and mucus are the point of entry and also the site for infection. In addition to the well-known diseases described below, there are many cases where the cause is unknown, for example red mark syndrome and warm water strawberry disease (Oidtmann *et al.* 2013).

Winter ulcer is a disease mainly affecting salmonids in sea water at low temperatures. The bacteria *Moritella viscosa* is considered to be the main causative agent (Karlsen *et al.* 2012), although other bacteria like *Tenacibaculum* spp and *Alivibrio wodanis* may be involved (Bornø and Lie 2014, Olsen *et al.* 2011). Infection gives extensive ulceration and fin rot, and can lead to reduced osmoregulatory capacity and increased susceptibility to other infections (Bruno *et al.* 2013). Winter ulcer represents the main bacterial infection in Norwegian aquaculture (Bornø and Lie 2014, Løvoll *et al.* 2009). The disease represents a substantial welfare problem, in addition to causing major economic losses due to mortalities and downgrading at slaughter.

Flavobacterium columnare is the causative agent of columnaris disease. It can cause a chronic, ulcerative necrotic infection on the skin and gills of salmonids in freshwater (Staroscik and Nelson 2008). Outbreaks are often related to stress, including high temperatures and low levels of dissolved oxygen, and can cause high mortalities (Bruno *et al.* 2013). *Flavobacterium psychrophilum* has also been described in wounds from rainbow trout and salmon in brackish water (Bornø and Lie 2014).

Ichthyophthirius multifilis is a protozoan ectoparasite (ciliate) known to cause white spot disease or "Ich" in freshwater fish (Ullal and Noga 2010). Infected fish present numerous white spots on the skin, dark coloration, excessive mucus and scale loss, and eventually dermal necrotic lesions (Bruno *et al.* 2013, Noga 2010). The parasite feeds on the skin mucus and epithelium, impairing gas and ionic exchange, finally causing mortalities. Fish that survive sublethal parasite exposure become resistant to subsequent challenge. This resistance correlates with the presence of humoral antibodies in the serum and cutaneous mucus of the fish, suggesting that mucosal antibodies are produced locally in skin (reviewed by Esteban 2012).

1.7 Ectoparasites (sea lice)

Several ectoparasites can cause problems for salmon farming in open cage systems. By far the most problematic parasites are copepods of the family Caligidae, collectively known as sea lice. The two species *Lepeophtheirus salmonis* Krøyer 1837 and *Caligus elongatus* Nordmann 1832, are the most important in salmon farming. *L. salmonis* is often referred to as the salmon louse because it is specific to salmonids, and especially to Atlantic salmon, while *C. elongatus* is less specific and has been reported from 80 different species (Boxaspen 2006).

The salmon louse has been a problem for the industry since the 1970s (Brandal and Egidius 1979), and is the most serious parasitic infection in salmon farms in the Northern Hemisphere (Pike and Wadsworth 1999). Although the parasite naturally occurs on

salmon in sea water intensification of farming has led to an increase in the number of natural hosts for the louse. Salmon lice have large economic consequences for the salmon industry, both as direct costs for the prevention and treatment, but also indirectly through negative public opinion. An estimate shows that sea lice cause economic losses for the industry of around 6% of the production value every year (Costello 2009).

In recent years the dependency on a limited number of therapeutics has led to a reduction in sensitivity to sea lice treatments (Bravo *et al.* 2008, Igboeli *et al.* 2012). Integrated pest management (IPM) strategies have been implemented in all sea lice affected regions (Brooks 2009), making sure that all available tools are being used to keep the lice number below levels where it has environmental or economic impact. IPM includes functional feeds that aim to lower the number of lice attaching to the fish. Altering the mucous layer through functional ingredients to make the mucous less suitable for the lice could provide a promising route for reducing parasite infestation.

The *L. salmonis* life cycle, as described by Johnson and Albright (1991), has two free living naupliar stages followed by a free-swimming infectious copepod stage. Thereafter follow four chalimus stages, two pre-adult stages and one adult stage (fig 3). Although this description has been the standard, new research describes only two chalimus stages (Hamre *et al.* 2013). Once the copepod has located a host, it establishes itself using the second antenna and maxillipeds to maintain its position (Bron *et al.* 1993, Bron *et al.* 1991). It is suggested that chemoreception is important for deciding whether the host is suitable (Fast *et al.* 2003, Ingvarsdóttir *et al.* 2002). The copepod begins to feed of the host, before it molts into the first chalimus stage. In the chalimus stages the louse is attached to the host by a frontal filament (Johnson and Fast 2004). The filament is renewed for each molt, giving the louse a possibility to reposition. This may be an advantage in avoidance of host immune response (Johnson and Fast 2004).

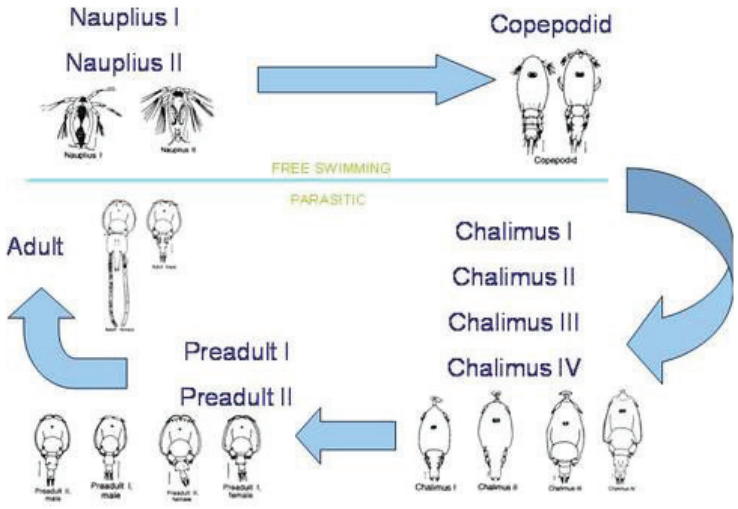

Figure 3. Life cycle of the salmon louse (*L.salmonis*)
(http://ookaboo.com/o/pictures/picture/13128473/Sea_louse_life_cycle) (modified from Schram (1993)).

All the developmental stages of sea lice that are found on the host feed on mucus, skin and/or blood (Fast 2014). This means that severe infections can remove the mucus and skin to expose the underlying muscle. This can again lead to reduced appetite, osmotic problems, secondary infections and mortality (Nolan *et al.* 1999, Pike and Wadsworth 1999). As with other biological processes the hatching of eggs are slower at low temperatures, making the sea lice problem less severe during winter.

Both epidermis and mucus can protect fish from the parasite, and clear differences have been shown in susceptibility between species. Fast *et al.* (2002b) measured several non-specific humoral parameters in rainbow trout, coho salmon and Atlantic salmon and found that the latter had the lowest activity of mucous lysozyme and protease, thinnest epidermis and sparsest distribution of mucous cells. Variations in these parameters are likely to have an effect on the response to sea lice infestation, explaining why Atlantic salmon are more susceptible to lice than the other two salmonid species. The sea lice

itself produce secretory products that affects the host. Fast *et al.* (2003) found that sea lice secrete more low molecular weight proteases on Atlantic salmon compared to coho and other marine species. This suggests that the less susceptible species might block the production of these proteases by the sea lice, while more susceptible hosts stimulate the production. Sea lice has also been shown to induce secretion of Prostaglandin E2 (Fast *et al.* 2004), a potent vasodilator thought to aid in parasite evasion from host immune responses. A component of the variability among host species depends on the extent to which inflammatory response is elicited by the louse attachment and feeding behavior (Braden *et al.* 2012, Fast *et al.* 2002a).

Several interacting factors can influence the host's susceptibility to sea lice infestation, including the host stress and nutritional status, the effectiveness of the host immune system and genetically determined resistance (MacKinnon 1998). Anti-parasitic vaccines has also been attempted for sea lice (Carpio *et al.* 2011), but do not seem to be relevant for the foreseeable future. The most relevant solution is a combination of methods, with immunostimulation of the host through feed additives in combination with appropriate therapy. This includes correct use of existing treatments and novel therapeutics, and improved genetic resistance through selective breeding.

1.8 Nutrition and skin health

Nutritional status is considered as one of the important factors that determine the ability of fish to resist disease (Gatlin III 2002, Lall 2000). For healthy skin all essential nutrients (proteins, amino acids, essential fatty acids, vitamin and minerals) must be sufficiently supplied through diet. It is not always known if the established requirements will cover the need under challenging conditions. Using novel feed ingredients in modern aqua feeds may also introduce uncertainty in inherent nutrient composition and nutrient bioavailability (Waagbø 2008). Optimal wound healing requires adequate nutrition, and malnutrition has been related to decreased skin strength and increased infection rates in

humans (Stechmiller 2010). Health diets, or functional feeds, include functional ingredients that promote health related functions, including antioxidants, vitamins, immunostimulants, organic acids and plant extracts. Feeding with functional ingredients can, in some instances have a systemic effect on immune functions but also on the fish integument, thus strengthening the skin and the mucus layer. These compounds are primarily aimed at strengthening the innate immune response, and have a general preventative effect against external stressors (Bricknell and Dalmo 2005, Kiron 2012, Sakai 1999).

Vitamin C plays an important role in the health status of fish. It is a strong reducing agent, and is also essential for collagen formation and wound healing (NRC 2011, Wahli *et al.* 2003). In humans, vitamin C is an important aspect of nutrition in wound management, as local concentrations quickly becomes depleted after skin injury (Landsdown 2004). High doses of vitamin C have been shown to increase resistance to several pathogens in fish (Navarre and Halver 1989, Verlhac and Gabaudan 1997, Waagbø *et al.* 1993). The proposed mechanism of action varies, but vitamin C has been shown to stimulate serum haemolytic complement activity, lysozyme, proliferation of immune cells, the release of signal substances and antibody production (Verlhac and Gabaudan 1997, Waagbø 2006, Waagbø *et al.* 1993).

Other vitamins may also have an impact on skin health. Deficiencies of vitamin A and several B vitamins can cause dark skin coloration and lesions, but this is not common as formulated diets for salmonids are supplemented with a balanced vitamin premix. Vitamin E is known as a powerful anti-oxidant, and is also shown to have impact on the non-specific humoral immune factors as well as the cellular response (Pohlenz and Gatlin III 2014, Waagbø 2006, Waagbø 1994).

Trace elements such as zinc, iron, copper and selenium are vital for maintenance of cellular functions in the immune system of higher vertebrates (Lall 2000). Zinc (Zn) has been shown to exert a beneficial effect on wound healing by modulating inflammation

and speeding up the re-epithelialization process in mammals. It stimulates the proliferation of keratinocytes and fibroblasts in wounds and increases collagen synthesis (Tenaud *et al.* 2000, Tenaud *et al.* 1999). In fish nutrition, Zn is one of the most important trace elements, as it is involved in various metabolic pathways (Watanabe *et al.* 1997). Deficiency of Zn has been associated with reduced growth, delayed wound healing and erosion of the fins and skin (Fountoulaki *et al.* 2010, Ogino and Yang 1979). Low fish meal and high levels of phytic acid present in plant raw materials increases the need for Zn supplementation in commercial fish feeds (Fountoulaki *et al.* 2010, Prabhu *et al*. 2014, Watanabe *et al.* 1997). Macrominerals, in particular calcium and phosphorus are essential for the calcified matrix of forming scales, and is therefore important for the re-growth of scales in the wound healing process (Vieira *et al.* 2011).

Yeast ß-glucans are probably the most used immunostimulants in fish feed. In nature, β-glucans are widespread and have been characterized in microorganisms, algae, fungi and plants. The products known as β-1.3/1.6-glucans, derived from baker's yeast, are suggested to be the most potent immune-system enhancers (Ringø *et al.* 2010). They have been proven to be potent activators of non-specific mechanisms of antibacterial defences in fish (Uribe *et al.* 2011). In aquaculture, ß-glucans have been successfully used to enhance the resistance of fish and crustaceans against bacterial and viral infections (reviewed by Dalmo and Bøgwald 2008, Ringø *et al.* 2012). In common carp, ß-glucan enriched baths seemed to promote wound healing, and to induce a local change in cytokine expression (Przybylska-Diaz *et al.* 2013). Marel *et al.* (2012) found that β-glucans increased the expression of a mucin and two β-defencin genes in carp mucus, showing that the mucosal system can be influenced by feeding β-glucans. The effect on parasites is not well-documented, but a reduced sea lice burden has been described in salmon fed a diet with β-glucans (Refstie *et al.* 2010, Ritchie 2000).

Nucleotides are building blocks of tissue RNA and DNA and of ATP (Ringø *et al.* 2012). Studies with dietary supplementation of nucleotides in fish feeds indicates a positive influence on disease resistance, sea lice infection, immune responses, stress tolerance,

vaccine efficacy, osmoregulatory capacity at transfer and growth rates in salmon (Burrells *et al.* 2001a, Burrells *et al.* 2001b). A review by Li and Gatlin III (2006) states that nucleotides may have an effect on both the innate and the adaptive immune system, but further research on the topic is necessary. So far, a direct effect of nucleotides on skin condition or mucus production has not been documented.

Probiotics are defined as substances or organisms that contribute to the intestinal microbial balance (Uribe *et al.* 2011). The beneficial effect of probiotics does not need to be limited to the gut, and new definitions describing the beneficial effects on health of the host are discussed (Kesarcodi-Watson *et al.* 2008). Suggested benefits of supplements includes growth promotion, inhibition of pathogenic microorganisms and augmentation of the immune response (Uribe *et al.* 2011). Probiotics may also stimulate the innate immune defence through TLR, other cellular receptors or humoral factors (Magnadottir 2010). Hernandez *et al.* (2010) found an increased level of protein in skin mucus of the Porthole livebearer (*Poecilopsis gracilis*), indicating an activation of mucosal response after feeding with probiotics.

Finally, plant extracts have been the subject of numerous studies, and are reported to have various effects like growth promotion, antibacterial, anti-parasitic, immuno-stimulant, anti-inflammatory and anti-stress (reviewed by Bulfon *et al.* 2013, Reverter *et al.* 2014). There is a growing body of evidence indicating that essential oils can have a function as control agents against a range of ectoparasites in a variety of animals (Ellse and Wall 2013). Also in fish there is evidence that plant extracts can have anti-parasitic effects, and synergetic effects of different extracts are described (Reverter *et al.* 2014).

It is clear that the interrelationship between nutrition and health is an important area that needs more investigation. Better understanding of how diet can support the integument and influence the mucosal immune response will lead to progress in the work towards improved skin health.

2. Aims of the thesis

The overall goal of the work was to improve the understanding of the condition of Atlantic salmon skin and its mucus layer under different conditions. The study aimed to investigate how the skin and the epidermal mucus layer were influenced by challenges encountered under normal farming conditions, and to assess the possibility to modulate the skin condition and response through dietary means. Four objectives were set:

1. Develop and optimise methodologies (quantitative histology, proteomics and transcriptomics) to assess the condition of salmon skin and mucus.
2. Assess the response of skin and mucus to environmental challenges (temperature changes) and validate the methods.
3. Study the effect of dietary modulation (vitamin C, vitamin E and Zn) on the skin and mucus under challenging farming conditions and after physical injury.
4. Study the effect of dietary modulation (immunostimulants, plant extracts) on the skin and mucus ability to resist/respond to parasite infection.

3. Abstract of papers

3.1 Paper I

Investigating the underlying mechanisms of temperature related skin diseases in Atlantic salmon, *Salmo salar* L.: as measured by quantitative histology, skin transcriptomics and composition

Skin integrity is recognised as of vital consideration for both animal welfare and final product quality of farmed fish. This study examines the effects of three different rearing temperatures (4, 10 and 16°C) on the skin of healthy Atlantic salmon post-smolts. Changes in skin condition were assessed by the means of skin composition analyses, quantitative histology assessments and transcriptome analysis. Level of protein, vitamin C and vitamin E were significantly higher at 16°C compared to 4°C. Quantitative histology measurements showed that the epidermal thickness decreased from low to high temperature, whereas the epidermal area comprising mucous cells increased. The difference was only significant between 4°C and 16°C. Both high and low temperature exhibited significant changes in the skin transcriptome. A number of immune related transcripts responded at both temperatures. Contrary to well-described immunosuppressive effects of low water temperature on systemic immunity, a subtle increase in skin mediated immunity was observed, suggesting a pre-activation of the mucosal system at 4°C. Up-regulation of a number of heat shock proteins correlating with a decrease in epidermal thickness suggested a stress response in the skin at high temperature. The results demonstrate distinctive temperature related effects on the skin of Atlantic salmon.

3.2 Paper II

Effect of temperature and diet on wound healing in Atlantic salmon (*Salmo salar* L.)

Compromised skin integrity of farmed Atlantic salmon, commonly occurring under low temperature and stressful conditions has major impacts on animal welfare and economic productivity. Even fish with minimal scale loss and minor wounds can suffer from secondary infections, causing downgrading and mortalities. Wound healing is a complex process, where water temperature and nutrition play key roles. In this study, Atlantic salmon (260 g) were held at different water temperatures (4 or 12°C) and fed three different diets for 10 weeks, before artificial wounds were inflicted and the wound healing process monitored for two weeks. The fish were fed either a control diet, a diet supplemented with zinc (Zn) or a diet containing a combination of functional ingredients in addition to Zn. The effect of diet was assessed through subjective and quantitative skin histology and the transcription of skin-associated chemokines. Histology confirmed that wound healing was faster at 12°C. The epidermis was more organized, and image analyses of digitised skin slides showed that fish fed diets with added Zn had a significantly larger area of the epidermis covered by mucous cells in the deeper layers after two weeks, representing more advanced healing progression. Constitutive levels of the newly described chemokines, herein named CK 11A, B and C, confirmed their preferential expression in skin compared to other tissues. Contrasting modulation profiles at 4ºC and 12ºC were seen for all three chemokines during the wound healing time course, while the Zn-supplemented diets significantly increased the expression of CK 11 A and B during the first 24 hours of the healing phase.

3.3 Paper III

Proteomic analysis of epidermal mucus from sea lice infected Atlantic salmon (*Salmo salar* L.)

Health diets that contain immunostimulants and other functional ingredients can strengthen the immune response in Atlantic salmon (*Salmo salar*) and thereby reduce the sea lice (*Lepeophtheirus salmonis*) infection levels. Health diets can be used as a supplement to other treatments and will potentially reduce the need for delousing and other treatments.

A sea lice infection trial was conducted on fish with an average weight of 215 g. One control diet and four experimental diets containing functional ingredients were produced. The diets were fed to salmon for 4 weeks before infection with sea lice copepodids. When lice had developed to chalimus III/IV, 88 fish per diet were examined for lice loads. Mucus samples from fish fed the different diets were taken before and after lice infection.

Mass spectrometry based proteomics was used to characterise the protein composition in the epidermal mucus of Atlantic salmon and to identify quantitative alterations in protein expression. Multivariate analysis of the generated data sets was performed to identify protein biomarkers. Putative biomarkers associated with functional feed intake and with sea lice infection have been identified and can form the basis for strategic validation experiments with selected functional feeds.

3.4 Paper IV

Reducing sea lice (*Lepeophtheirus salmonis*) infestation of farmed Atlantic salmon *(Salmo salar* L.) through functional feeds

Health diets for Atlantic salmon have become an important component of the Integrated Pest Management strategies targeting sea lice. A challenge trial was performed in order to examine the effect of supplementing salmon diets with either immunostimulants or essential oils.

One control and four experimental diets containing immunostimulants or natural identical extracts were fed to Atlantic salmon in triplicate tanks for 4 weeks before challenging the fish with the sea lice copepodids. Prevalence of infection was 100%, and the mean abundance of infection was 21.2. The lowest mean lice count of 17 per fish ($p<0.05$) was found in the group fed a mix of natural identical plant extracts (PX I). This represents a 20% reduction in infection, showing the potential for health diets to be employed as a tool to help control sea lice.

To gain an understanding of the mechanisms of action underlying this protection, fish fed the control diet and fish fed the PX I diet were compared using quantitative histology of the epidermis and proteomic analysis of epidermal mucus. No significant differences were seen in the thickness of the epidermis or mucous cell percentage area, but differences in expression was seen for a number of proteins, including heat shock proteins, in epidermal mucus

4. Methodological considerations

4.1 Experimental conditions

The fish trial in Paper I was run at Stiftelsen Industrilaboratoriet (ILAB) in Bergen (Norway), the challenge trial in Paper IV was carried out at the Marine Environmental Research Laboratory (MERL) in Machrihanish (Scotland) and the experiments in Paper II and III were run at Lerang Research Station, the R&D facilities of Skretting ARC for salmonids located in Stavanger (Norway). ILAB and Skretting ARC have long experience in running feeding trials with salmon, and the trials in Paper I and Paper II were executed without any complications.

High quality experimental facilities and validated methods for rearing fish and lice are crucial for conducting challenge trials with sea lice. The sea lice challenge trials in Paper III and IV were performed in a similar manner at the two facilities. The main difference was the lower number of copepods per fish in Paper III, and the fact that in this trial the water level and circulation remained unchanged during the infestation process. The outlet was covered with a 100 µm fine mesh for 2 hours after adding the copepods. In Paper IV the level of water in the tank was reduced and the water circulation stopped for 3 hours. This may be part of the explanation for the overall higher lice count at MERL.

The lice strains used in the two papers were also different. The lice used at MERL were from a naïve strain collected from a farm site in 2001 and cultured in the laboratory since. The lice had been cultured without any exposure to chemical lice treatment. The MERL has long experience in running challenge trials with sea lice (see e.g. Stephenson 2012, Bassett *et al.* 2012, Carmichael *et al.* 2013). In paper III the strain used was LsGulen, collected, reared and tested by the University of Bergen (UIB) (Hamre *et al.* 2009). This strain is non-resistant to current commercial anti-sea lice treatments. The egg strings were sampled at ILAB and stored overnight in 9°C sea water. Next day the egg strings were transported to Lerang, and transferred to a hatching cylinder inside the sea lice facility

(fig 4). The transport was optimised to avoid that changes in water quality impaired the lice vitality, and consequently their ability to attach to fish.

In both experiments the number of sea lice per fish was determined at the chalimus stage 12 days after infestation, under a dissection microscope. Counting lice is a time consuming exercise and requires training of the involved personnel. Since lice display individual differences in their developmental rates, the pre adult stage, which lacks the frontal filament, can occur at a similar time as the chalimus. Pre-adult lice may fall off the fish due to handling and anesthesia, with the resultant risk of not being counted. Therefore all the fish from each tank were collected and kept in separate containers. The lice remaining in the containers were counted after all fish had been sampled.

Figure 4. Sea lice experimental facilities at Lerang Research Station. a) Cylinder for hatching egg strings and rearing from nauplia to the infective copepodid stage b) Dedicated challenge unit for sea lice infection trials with Atlantic salmon.

Although there is no recognised standard procedure for how to infect salmon in tanks with sea lice, the method used in Paper III and IV are in accordance with several published challenge trials (Easy and Ross 2009, Fast *et al.* 2002a, Gjerde and Saltkjelvik 2009, Mustafa *et al.* 2000, Skugor *et al.* 2008). Although the methodology was similar,

most of the cited publications used higher number of copepodids, resulting in high numbers of chalimus stages on the fish. It has been debated whether such high lice numbers are representative of the natural farming situation (Wagner *et al.* 2008), but low numbers can make it difficult to uncover significant differences between groups, in part due to individual fish variation or lice burdens. To ensure statistically robust and comparable data, infection variables such as abundance and intensity should be standardised (Wagner *et al.* 2008). The functional ingredients tested in the diets were not medication, and thus it would become difficult to show differences in lice numbers if the lice burden were too high. In paper III and IV, the sea lice challenge was used as a model to assess any impact of dietary modulation on the mucus or skin of the fish. Since the diets tested in the two experiments were different, there is no direct comparison between lice numbers in the trials, and the variation in methodology is not critical.

4.2 Quantitative histology

Despite the increasing use of new molecular techniques in pathology, conventional histology is still routinely employed to monitor tissue structure alterations. Histological evaluation of fish skin has traditionally been described by qualitative assessment, where typical changes in skin structure have been described after stress induction (Iger *et al.* 1995, Iger and Wendelaar Bonga 1994, Roberts *et al.* 1973, Roberts *et al.* 1970). The methods for assessing lesions are rather divergent among different authors. Some of them describes the changes morphologically (Mitz and Giesy 1985) whereas others assess the structural changes on an arbitrary scale (Bucher and Hofer 1993). The use of different methods and measurements makes it difficult to compare the changes observed, and more standardised methods are needed (Bernet *et al.* 1999). The use of histopathology alone relies on the visual analysis of morphological changes, and is therefore prone to subjective interpretation. Traditional histopathology also depends on experience and recognition skills of the pathologist, and can be difficult to reproduce (Jarvis 1992). A

quantitative measurement of histological data would remove part of the subjectivity and make data more reproducible.

Importance of sampling technique, processing and staining. In paper I, II and IV the samples for histology were taken from standardised areas as described in the publications. Care was taken to avoid touching or compromising the sample area before and under dissection, and all samples were immediately fixed in 4% phosphate buffered formalin. In Paper IV the processing and staining was performed at the Stirling University (Institute of Aquaculture, Histopathology Laboratory) as described in the publication. For paper I and II the fixated samples were processed and stained at the University hospital of Stavanger (SUS), before they were scanned and analysed (see fig 5 for work flow). Transverse sections of the skin were chosen in all the experiments to get an overview of all the skin layers, and to be able to assess the variation in epidermal thickness. Transverse sections also allow for determination of mucous cell position in the epidermis, and enables potential assessment of migration of mucous cells in response to stress. When investigating the difference between tangential and transverse sections the average cell area and the ratio of epidermal to mucosal tissue were similar between the two sections (Pittman *et al.* 2011).

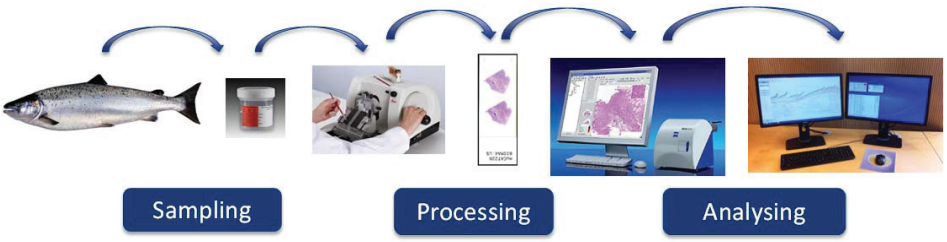

Figure 5. Work flow from sampling fish skin to quantitative analysis.

Transversal sections will cut through a random point of the mucous cell, sometimes in the tip of the cell and other times in the centre. This could generate errors when assessing cell size if the size of the sample analysed is too small, but will be evened out by analysing

large sections of epidermis using quantitative measurement tools. In the different papers, the areas of epidermis covered by mucous cells rather than the number of cells were chosen as the method to compare samples. This makes it possible to compare results between body locations, individual fish and treatments.

Staining. Standardisation of the staining technique is crucial when employing automated image analysing. The software is sensitive to even small differences in colour, and development work on staining techniques was performed to optimize the quality. Several standard and special stains were tested (fig 6), and the most consistent and interpretable results were obtained with Alcian Blue/Periodic acid – Schiff (AB/PAS) special staining kit as described in Paper I. AB/PAS is a stain developed for the study of both acidic and neutral mucins (Mowry 1956), and gives a clear separation of the mucous cells from the background (fig 6f). The variability between batches was minimised by using an automated Bench Mark special stainer (Roche).

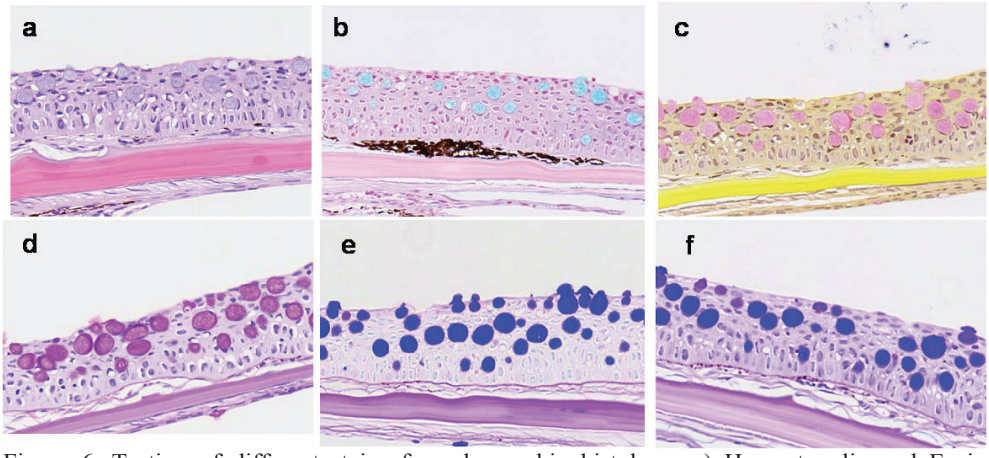

Figure 6. Testing of different stains for salmon skin histology. a) Haemotoxylin and Eosin (H&E), b) Alcian Blue (AB) c) Mucicarmine d) Periodic acid-Schiff reaction (PAS) with diastase e) AB/PAS and f) AB/PAS H

Image acquisition and analysis. Stained sections from Paper I and II were scanned on a single slide scanner (Mirax Desk, Carl Zeiss, GmbH). This technology allows for high resolution images to be obtained. Digital slides have several advantages compared to

glass slides, but the high resolutions needs to be compromised with processing time requirement, image quality and file size (Da Silva 2013). The quality of the scanned images was checked in Pannoramic viewer (3DHISTEC Ltd.) before being subjected to quantitative analysis using Visiopharm (VIS) 4.6 software (Visiopharm, DK), in the module Visiomorph.

Regions of interest (ROI) were manually drawn over an area of the epidermis covering as much of the samples as possible, avoiding the tip of the scale and damaged areas. The ROIs were visualized as a surrounding line overlaying the virtual image. Different color de-convolution bands were previewed on a selection of images to define the best differentiation between the staining colors. A custom-made colour de-convolution was then applied to preprocess the images. The purpose of preprocessing is to make it easier for the classifier to perform image segmentation. Preprocessing can either enhance or remove a particular phenomenon that will improve or disturb the subsequent image segmentation. Preprocessing steps includes single pixel operations, using the value from one pixel to create a new value for that same pixel, and filter operations, using the values from a range of neighboring pixels to create a new value for a single new pixel. Standard filters can be used to remove noise, unify areas and to improve contours of morphological structures. After preprocessing, a Bayesian classification was applied. This classification requires training of the program, where manually painted labels on the image are defined by the user. Each label represents pixels with similar features, and must be repeated for all features the user wants to differentiate. The training was then tested on a montage of images, and the steps were repeated until the classification was in line with the underlying features. The result of the image segmentation is then a set of labels that collectively covers the whole area within the ROI. All pixels belonging to a specific label are then equal with respect to the defined characteristic such as colour, intensity or texture.

Post processing steps and calculations. After the classifier had segmented the image into classes visualized by labels of different colors overlaying the image, a series of post

processing steps were applied. Even with successful preprocessing and classification minor adjustments may be necessary to clean up the image. Main steps were performed to remove noise, separate adjacent mucous cells, fill holes in tissue or unify objects in order to get an image segmentation where correct calculations could be performed. Numerous post processing possibilities to fine tune the segmentation result are available, but need to be used with care to avoid erroneous results. Subjective control of all images and rigorous testing of the automated classifier are key elements for correct quantification. All calculations were based on the output after post processing.

Description of the two developed protocols. In the first protocol the mucous cells were individually separated from the epidermal area, and then labeled according to their distance to the skin surface (see Paper I, Fig 1). The mucous cell area fraction, of either all mucous cells or only mucous cells within a certain threshold distance from the surface, could then be calculated by the following formula:

$$Mucous\ cell\ area\ fraction\ (\%) = \frac{Tissue\ area\ comprising\ mucous\ cells}{Total\ tissue\ area} * 100$$

The second protocol, developed to calculate the epidermal thickness, was based on the image segmentation from the first protocol. In Paper I, using further post processing steps, skeletonization was used to create a line through the middle of the epidermis (see Paper I, Fig 1). Half of the interface length between this line and the epidermal layer represented the length of the epidermal layer. The average epidermal thickness was then calculated by dividing the epidermal area by the length of the epidermal area. In Paper II a slightly different method was used to measure thickness. Again, through a series of post processing steps, the mucous cells were unified with the epidermal area, thus creating an interface only between the epidermal area and the external environment. The length of this interface estimates the length of the epidermal area. The average epidermal thickness is then calculated by dividing the epidermal area to the length of the epidermal area:

$$Average\ epidermal\ thickness\ = \frac{Epidermal\ area}{Length\ of\ epidermal\ area}$$

Output. When the protocols were completed, the designated slides were selected and the protocol set to batch process. The protocols for mucous cells and epidermal thickness were run separately, and the output calculations were exported to an excel file. It was possible to output number, size and range of data from all objects within each classification. The intensity and position of every pixel was known, making possibilities for numerous calculations such as intensity within each class, measures of shape and size, numbers of objects and area fractions. This could be calculated in the classifier or after data export to excel or any suitable statistical software. For validation a manual control was conducted on each slide after running both protocols. By changing the transparency of the labels it was possible to control if they were correctly overlaying the morphological structures in the digital image as specified in the protocols. Epidermal thickness, numbers, diameter and area of mucous cells were also controlled manually (fig 7).

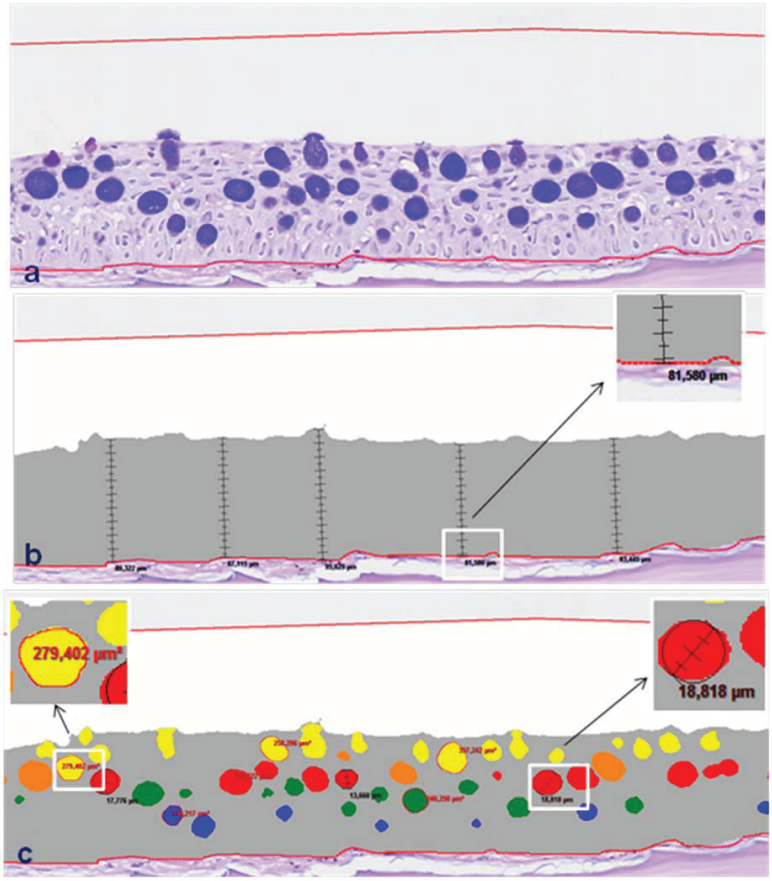

Figure 7. Measurements of skin epidermal thickness, from the automated protocol were validated by taking numerous manual measurements a) Epidermis with mucous cells (stained dark blue) b) Repeated manual measurements of epidermal thickness c) Measurements of mucous cell diameter (µm) and area (μm^2).

4.3 Proteomics

Proteomics is one of the novel 'omics' technologies, that allows for large number of genes, transcripts, proteins or metabolites to be examined in a single analysis (fig 8).

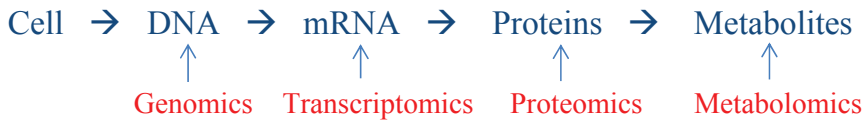

Figure 8. Schematic overview of the different 'omic' sciences.

Proteomics can be defined as the study of the complete set of proteins expressed by a cell or an organism, including their structure and function. The proteome represents the entire collection of proteins present in a cell, tissue or fluid in a living organism at a given time point. The proteome is very dynamic and can therefore be more challenging to study than the genome. As the functional units of the cells are proteins, and not the mRNA, it can be more relevant to study changes in protein expression than changes in the transcriptome, to reveal the impact on external factors including diet and parasitic infestation.

Proteomic techniques are usually divided into gel-based (e.g. two dimensional (2D) gel electrophoresis) and gel-free approaches. 2D gel electrophoresis has been the most used approach in proteomic research (Görg *et al.* 2004), but in the recent years gel-free mass spectrometry (MS)-based methods have become the method of choice for both identification and quantification of proteins (Nesvizhskii 2010). MS-based proteomics are proposed to be more sensitive than 2D gel electrophoresis, although in characterization of specific proteins, these two techniques are increasingly used in conjunction (Oliveira *et al.* 2014). MS based proteomics are now increasingly applied in the context of systems biology studies, where it is used in parallel with other technologies like gene expression analysis and metabolomics.

In paper III and IV the liquid chromatography tandem mass spectrometry (LC-MS/MS) shotgun approach to proteomics was used to characterise the epidermal mucus from

salmon. In Paper IV the mucus samples were gel fractionated before further analysis, and the two fractions with highest content of proteins were chosen for the LC/MS-MS. This resulted in fewer proteins in the following analysis. The Shotgun proteomics refers to the bottom-up method, where proteins are digested by proteases (e.g. trypsin) into peptides, and separated by LC prior to MS/MS analysis and characterization (Nesvizhskii 2010, Yates *et al.* 2009). The first MS analyses the mass to charge ratios (m/z values) of all peptide ions. Selected peptide ions are then fragmented into smaller size in the collision cell of the MS instrument. The second MS separates the fragmented products according to mass. This MS/MS spectrum allows identification of the amino acid sequence of the peptide that produces it (Nesvizhskii 2010).

The accuracy of the instrument does have impact on the analysis. The LTQ-Orbitrap is known to give high quality output, and has become a very powerful tool in proteomic research. It has been shown to have high mass accuracy, high resolution, large ion capacity, and large dynamic range (Han *et al.* 2008, Yates *et al.* 2009). Even with high mass accuracy instruments, the accuracy depends on fine tuning and regularly calibrations (Nesvizhskii 2010). The analyses were therefore overseen by dedicated operators at IRIS, and the instrument was calibrated weekly as recommended by the manufacturer.

Data treatment

Proteomics produces high out-put of data, and the scientific outcome is completely reliant on robust analysis of the experimental data. Good tools for statistical analyses reduce the risk for false positive output (Nesvizhskii 2010, Slattery *et al.* 2012).

Databases provides matches for almost every input MS/MS spectrum, but only a fraction of those peptide to spectrum matches (PSMs) are true (Nesvizhskii 2010). Statistical validation of the PSMs is therefore crucial, and the score of the best match is converted into a p-value. To calculate statistical measures for large collections of PSMs, additional modelling is required. Peptide identification can be performed by correlating acquired

experimental MS/MS spectra with theoretical spectra predicted for each peptide contained in a protein sequence database.

The raw data from the LC-MS/MS were analysed using Proteome Discoverer 1.3 (Paper III) or 1.4 (Paper IV) with the Sequest algorithm. The Sequest search algorithm correlates experimental MS/MS spectra through comparison with theoretical in silico peptide candidates derived from protein databases. Proteome discoverer 1.4 has a decoy database search feature to determine False Discovery Rates (FDR). The FDR is a statistical value that estimates the number of false positive identifications among all hits found by a peptide identification search.

In both paper III and IV the MS/MS spectra were initially searched against the Salmonidae database (UniProt, www.uniprot.org). In paper IV a decision was made to search the Atlantic salmon database available from UniProt, although the genome database is not yet completed. This means that only peptides with previously identified spectra that are entered into that library could be identified. The decision to search the Atlantic salmon database from Uniprot was made as this database is comprehensive, and limiting the search to one species means that identification of the same protein with different entry numbers would be reduced. The risk of using an incomplete database is that all proteins in the sample may not be identified. 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 salmon genome is fully annotated and available, it will provide very relevant information for future proteomic studies.

4.4 Transcriptomics

Gene expression analysis

The translation process of a gene to its respective protein is called gene expression, where the transcription of the coding regions (exons) of DNA into mRNA is the first stage. The total set of mRNA produced by a cell or a population of cells at a certain time point can be defined as the transcriptome. Various methods have been developed to analyse the transcriptome (i.e.transcriptomics), with microarray based gene expression profiling being one of the most applied approaches. Microarrays can be used to screen for expression levels of large number of genes, thus producing copious gene expression data. The analysis itself is a complex, multistep technique with potential sources for both technical and biological variation. To obtain meaningful results, experiments must be carefully planned, protocols must be standardised and correct data normalisation and analysis methods must be applied (Jaluria *et al.* 2007, Quackenbush 2001). The microarray experiment in Paper I was therefore carried out in collaboration with specialists from the University of Stirling, and the Minimum Information About a Microarray Experiment (MIAME; Brazma *et al.* 2001) guidelines were followed. In the study the Salmon 4x44K Agilent oligonucleotide microarray was used. Although containing a large amount of information, the complete salmon genome was not available before the design of the microarray. In addition there have been very few studies of the skin transcriptome, so it is plausible that the existing arrays do not fully portray the skin transcriptome (Micallef *et al.* 2012). Thus, specific genes exclusively expressed in skin may not have been printed on the array.

Although widely used, the transcriptomics interpretation has some potential pitfalls. The obtained results are like snapshots, and only give information of the status of the cells or tissues transcriptome at one specific point in time. This description can be valuable, but it does not give information about mRNA transcription rate or turnover, or about protein synthesis. Changes to the transcriptome induced by experimental treatment is often time

dependent, and different genes may be regulated in contrasting directions. To reveal the true changes and find the peaks in expression level of a regulated gene, a time course study could be necessary. This was beyond the scope of the present thesis. It should be underlined that the microarray experiment in Paper I was intended to be a screening study for hypothesis generation, where the aim was to investigate any changes in the transcriptome due to temperature differences. No attempt to verify the findings with e.g. RT-PCR or Western blotting was conducted. The result was seen in correlation to the chemical composition and the histology results.

5. General discussion

The overall goal of this thesis was to improve the understanding of Atlantic salmon skin and mucus, and firstly targeted development and validation of methodologies to assess skin condition. These methods were discussed within chapter 4. Subsequent aims included implementation of the developed methods (quantitative histology, proteomics and transcriptomics) to assess the response of skin and mucus to an environmental challenge (temperature), physical injury (skin wounds) or a parasite infestation (sea lice). These findings will be outlined in the current chapter, with emphasis on the impact of these external factors on the skin chemical composition, structure and mucus composition (protein and gene expression). The final aim was to study the effect of dietary modulation under conditions representing challenging farming situations (skin wounds and sea lice infestation), and these results are discussed within chapter 5.4.

5.1 Chemical composition of salmon skin

At the start of this project, there were surprisingly few publications with primary focus on the chemical composition of fish skin tissue and mucus. To obtain more knowledge on the composition, a range of chemical analysis were performed on skin from salmon of around 200 g (Paper I). Temperature change was utilised as a relevant factor to study the impact of environmental stress, and the results demonstrated an impact on the chemical composition of the skin tissue. Protein level was significantly increased at high temperature, while the moisture level was reduced. This protein increase corresponded to an increase in the concentration of the three main amino acids in collagen: proline, glycine and hydroxyproline (Szpak 2011). The sum of these three amino acids constitutes 33% of the amino acids measured in the group kept at 10ºC, but reached 36% in the group reared at 16°C. This indicates a higher presence of collagen in the skin of salmon exposed to high temperatures. This increase in collagen most probably reflects a higher

physical strength of the skin as reaction to elevated temperature, along with adaptations in tissue structure.

The sum of fatty acid also varied between temperatures, with a higher level at 16°C than at 10°C. The difference was small, but still significant, and putatively related to lower digestibility of fat in salmonids at low temperatures (Ng *et al.* 2004, Olsen and Ringø 1998). In general, fat content is also known to increase with increasing fish size (Jobling and Johansen 2003), and a considerably higher level of fatty acids has been analysed in the skin of large salmon (2.9kg) (Aursand *et al.* 1994). Although the difference in size between the fish at 4°C and 16°C was small, it could have impacted on the fat level in the skin. The higher degree of fatty acid unsaturation in fish exposed to low temperature has been suggested to be partly responsible for maintaining proper cell membrane fluidity through homeoviscous acclimation, as a necessary adaptation for ectotherms exposed to low temperature (Bogevik *et al.* 2010, Farkas *et al.* 2001).

Vitamin C and E in skin both increased from low to high temperature. As vitamin E is fat-soluble, the higher level in skin could be related to the higher fat digestibility. Another factor influencing the vitamin results is that fish size and feed intake increased with increasing temperature, allowing larger fish at higher temperature to accumulate more vitamins. To avoid nutritional deficiency, commercial diets are supplemented with vitamin C and E to cover the nutritional requirements (NRC 2011), with conservative safety margins. To see an effect of different vitamin and mineral levels in the skin, it is likely that a more invasive stress than the current temperature change must be imposed, perhaps coupled with a marginal nutritional deficiency. Vitamin C and E have been found to modulate different parts of the immune system and prevent mortalities during disease outbreaks (see reviews by Waagbø 1994, 2006). The temperature difference in Paper I demonstrated effects on the chemical composition of salmon skin, but in this trial there was no sign of disease. Some pathologies affecting skin are temperature related (Le Morvan *et al*. 1998, Lillehaug *et al*. 2003), and this basic knowledge on how temperature

affects the composition of skin forms a good base for further studies. More information is needed on how skin composition is affected by fish size and disease outbreak.

5.2 Impact of exogenus factors on skin structure

As previously mentioned, the impact of three types of exogenous factors on skin structure has been tested and studied through histology in the different papers: an environmental factor (temperature), a pathological challenge (sea lice infestation) and a physical injury (skin wounding).

Papers I and II demonstrated that temperature had a significant impact on measurable skin histological parameters. In Paper I, the epidermal thickness decreased gradually from low to high temperature (fig 9a), whereas the epidermal area comprising mucous cells increased. Only the difference between 4°C and 16°C was significant, but a decrease in thickness and an increase in mucous cell area could also be seen at 10°C relative to 4°C. Across several studies, epidermal thinning in response to various stressors has been characterised as being an adaptive rather than a degenerative response (Iger *et al.* 1994b, Iger and Wendelaar Bonga 1994). Most studies focus on short term exposure to stressors (Iger *et al.* 1994b, Iger *et al*. 1994c), with evidence that the epidermis returned to normal thickness after the stressor had been removed. However, prolonged high water temperature has also been shown to lead to chronic epidermal thinning in the minnow (*Phoxinus phoxinus* L) (Bolognani-Fantin *et al*. 1984). In Papers I and II, the fish were kept at different temperatures throughout the trials, and recovery after returning to control condition was not studied. Salmon have a high temperature tolerance and are able to adapt to a range of temperatures (Elliott and Elliot 2010), so it is reasonable to assume that the changes seen in the epidermis are mainly adaptive. But then again both 4°C and 16°C could be outside the optimal thermal range for salmon, with potential impact on growth rates (Grini *et al*. 2011, Handeland *et al.* 2008). It is not possible to conclude whether the observed changes in epidermal thickness represents an adaptive response, or

an integrated stress response that could reduce the salmon's capacity to tolerate subsequent or additional stressors (Wendelaar Bonga 1977).

In unpublished data from Paper II, the skin histology of the fish fed the control diet at 4°C and 12°C degrees was assessed before eliciting standardised wounds. The results showed that the epidermis was significantly thinner at 12°C than 4°C preceding the trauma (fig 9b). The area covered by mucous cells was slightly greater at the higher temperature, but this was not significant. Higher temperatures were not assessed in this trial, so the reduction in epidermal thickness could only be described from 4°C to 12°C. This decrease was consistent with the findings from Paper I. The change in epidermal thickness at low temperature are probably more adaptive and regulated by factors distinct from those involved at high temperatures, as indicated by the skin mediated immune activity in the micro array data from Paper I (discussed in 5.3).

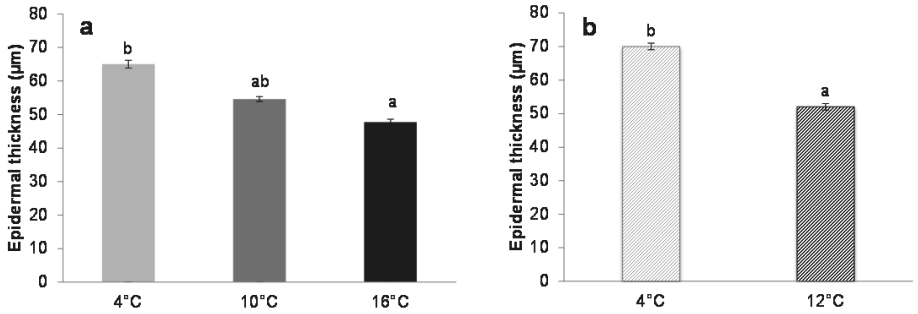

Figure 9. Epidermal thickness of Atlantic salmon from two trials at different temperature a) 4 and 10 and 16° C (fig. modified from Paper I) b) 4 and 12°C (unpublished data from Paper II). n = 10 per temperature. Superscript letters denotes significant differences.

Epidermal thickness has been proposed as a factor involved in the differential susceptibility to sea lice between salmonids. Fast *et al*. (2002a, 2002b) found that compared to rainbow trout and coho salmon, Atlantic salmon had thinner epidermis while also being more susceptible to lice. The same authors did not find any difference in thickness between control and infected fish in any of the species. In Paper IV and in

unpublished data from Paper III, epidermal thickness was assessed in fish challenged with sea lice at temperatures varying from 12ºC to 15ºC. Both experiments revealed no impact on thickness or mucous cell area before and after infection, or between diets (table 1). The stress of being infected with sea lice did not have the same influence on epidermal thickness as the temperature stress, meaning that the lice infection did not have a systemic effect on the assessed parameters.

Table 1. Epidermal thickness (mean ± sd) and area of epidermis comprising mucous cells (mean ± sd) from Paper IV and unpublished data from Paper III

Group	Area	Epidermal thickness (µm)	Mucous cell area (%)
Paper IV			
Control infected	Dorsal	61.0 ± 7.1	14.0 ± 4.0
PX I infected	Dorsal	57.8 ± 5.2	11.0 ± 3.0
Paper III, unpublished data			
Control uninfected	Dorsal	74.2 ± 8.0	7.0 ± 1.3
Control infected	Dorsal	78.9 ± 22.2	6.8 ± 3.1
Diet B uninfected	Dorsal	78.3 ± 15.2	7.2 ± 3.6
Diet B infected	Dorsal	76.4 ± 22.7	7.7 ± 4.1
Control uninfected	Head	40.5 ± 5.2	13.8 ± 4.1
Control infected	Head	39.7 ± 10.3	14.4 ± 3.3
Diet B uninfected	Head	42.6 ± 7.2	11.5 ± 6.0
Diet B infected	Head	41.0 ± 2.8	15.2 ± 3.8

None of the experiments showed any difference in epidermal thickness between fish fed control and the experimental diets that resulted in significant lower lice counts. This finding strongly indicates that the thickness of the physical barrier alone cannot explain the susceptibility to lice attachment. This is also supported by a recent study that compared salmon selected for high and low resistance to lice (Holm *et al.* 2015). The authors found a slightly (but not significantly) thicker epidermis in the putatively lower resistant group, but this did not seem to be related to the lice numbers.

It is known that heavy sea lice infections can cause severe damage to the fish by removing mucus and skin and exposing the underlying muscle (Fast 2014). Salmon

infected with as few as 3 to 10 pre-adult lice per fish have shown changes in the epithelial structure of the skin and gill (Nolan *et al.* 1999). This could be attributed to an effect of the immunosuppressive secretions of the lice, but also potentially related to the greater damage caused by pre-adult and adults compared to chalimus stages. In both Papers III and IV the fish were sampled when the lice had reached the chalimus stage, and it is likely that any changes in the epidermis would be induced at a later stage. In addition all samples were from standardized sampling points, and did not include the site of attachment. However, the local skin response in Atlantic salmon is generally lower grade compared to other less susceptible salmonids (Braden *et al.* 2012, Fast *et al.* 2002b, Johnson and Albright 1992). Epidermal hyperplasia and associated inflammation of underlying tissue is described in coho salmon within 1 day post-infection, but is absent in the Atlantic salmon (Johnson and Albright 1992). If the response is not systemic, it could be necessary to sample the area around the parasitic attachment site for future investigations.

In the two trials with sea lice (Paper III and IV), there was no difference in epidermal thickness between uninfected fish and fish infected with low or high lice burdens. As mentioned earlier, this suggests that epidermal thickness alone has no direct impact on lice attachment. Also the stress resulting from lice attachment seems to be differently regulated than temperature stress, where the latter results in a systemic change of epidermal thickness and mucous cell area (%). In general, the physiological mechanisms leading to alterations in epidermal thickness and mucous cell abundance are still not clear, and require further investigation.

5.3 Impact of exogenus factors on proteins and gene expressions

Changes in skin and mucus protein composition were studied as a consequence of changes in temperature (transcriptomics), as a result of skin wound infliction (transcriptomics) or sea lice infestation (proteomics) and after dietary manipulation (proteomics/transcriptomics). Due to the different methods used in each trial, the findings are not directly comparable, but some general conclusions can be drawn.

In Paper III, proteomic analyses were used to identify a total of 521 proteins in the epidermal mucus of salmon. The large number of proteins identified confirmed that mucus is an informative and complex matrix that could be useful in future studies. The number of proteins is similar to another gel-free proteomics study, in which 508 proteins were found in mucus from the gill and the skin of salmon (Valdenegro-Vega *et al.* 2014). In both Paper III and IV, a portion of the identified proteins in mucus were serum-derived proteins, like serum albumin and serotransferrin. These have also been identified in other studies of salmon mucus (Easy and Ross 2009, Valdenegro-Vega *et al.* 2014). Their presence is not fully understood, but leakage from the plasma or local secretion from the epidermis has been suggested (Valdenegro-Vega *et al.* 2014). The abundance of the serum proteins could be a limitation to the value of this approach, as they could possibly mask the presence of lower abundance native mucus proteins.

In the proteomics approach in Paper IV, the mucus was fractionated before the LC-MS/MS analysis, and in addition the Atlantic salmon genome database was search instead of the broader salmonidae database. This reduced the number of identified proteins (39-59 proteins), but assured that they were relevant for salmon therefore resulting in a decreased sensitivity but an increased specificity. Several of the proteins identified in the mucus samples in papers III and IV are associated with immune responses, stress responses and defence responses, indicating the importance of skin

mucus as a first line of defence in fish. A number of immune and stress related transcripts were also identified from skin in Paper I.

Heat shock proteins (HSPs) were originally described as inducible molecules capable of maintaining cellular homeostasis against abrupt temperature changes, but were later determined to represent an adaptive physiological response that copes with a variety of different proteotoxic stresses (Bottoni *et al*. 2009). HSPs are known to play an important role in aquatic animal health, in the host response to environmental challenges, toxins, and in particular in the development of inflammation and the specific and non-specific immune responses (Roberts *et al*. 2010). In Paper I, several HSPs were amongst the immune- and stress-related transcripts that responded to temperature. Up-regulation of HSPs at 16°C correlated with decreased epidermal thickness, suggesting a stress response at high temperature compared to low temperature as discussed earlier. HSPs have also showed increased expression in the skin of trout after short term exposure to high temperature (Burkhardt-Holm *et al.* 1998). Although HSP response has been considered to be a short-term response to stressors, it is becoming clear that the HSPs also play significant roles in regulating immune response in both mammals (Pockley 2003) and fish (Roberts *et al.* 2010, Xing *et al.* 2013). Since the up-regulation of HSPs was significant four weeks after increasing the temperature in Paper I, it is apparent that they do have some prolonged effect.

In Paper III and IV several HSPs were identified in mucus in the proteomic studies. The expression of HSPs varied between fish with high lice numbers and uninfected controls, and also between infected groups with low and high infection rate. Highly significant up-regulation of HSPs has also been described in damaged salmon skin after sea lice infection (Skugor *et al*. 2008). The authors propose that this up-regulation could be part of a process known as unfolded protein response (Malhotra and Kaufman 2007), typically seen in wounded tissue. A review by Roberts *et al*. (2010) discuss the HSPs roles in infectious diseases in fish and shellfish, describing a large variation in expression related to species, disease and timeline during infection. In paper IV, the expression level of

these stress-related proteins was reduced in fish fed the diet containing a mix of plant extracts (Diet PX I). This apparent down-regulation was probably related to the lower lice load in this group compared to the control, but could also be influenced by the dietary additives. This needs to be studied further, without the influence of lice on the fish. Another plant extract (*Opuntia ficus indica*) has been shown to induce expression of HSPs and reduce negative effects of cellular stress (Boerrigter *et al.* 2014). HSPs have also been used as markers of stress in nutritional studies (Bakke-McKellep *et al*. 2007, Sagstad *et al*. 2007). Despite the large number of studies focusing on HSPs, the understanding of them in relation to farmed fish is still limited. HSPs have potential as biomarkers of stress, but more knowledge is needed regarding the relationship between the cellular and physiological stress response (Iwama *et al*. 2004), and also on the response to sequential stressors.

Chemokines are another group of proteins with high potential as biomarkers for future skin research, due to their association with wound healing and tissue re-modelling (Gharaee-Kermani and Phan 2001, Gillitzer and Goebler 2001, Zaja-Milatovic and Richmond 2008). Several chemokines were upregulated in fish exposed to low temperatures, although these fish did not present any visible wounds (Paper I). The up-regulation of both chemokines and also interleukins was only in the range of 1.5 fold, but the subtle change in the immune activity seen in skin at 4°C suggests a pre-activation of the mucosal immunity at low temperatures. The expression pattern in salmon skin affected by winter wounds or mechanical damage shows a significantly higher up-regulation (30-fold), confirming their crucial roles in the wound healing process (Ingerslev *et al*. 2010). The salmon chemokines CK11A, CK11B and CK11C were preferentially expressed in the skin in comparison to other tissues as shown in Paper II, and their transcription levels clearly modulated both in response to wounding and dietary supplementation (Zn). Also in this trial a temperature effect was seen. Different modulation profiles were seen between 4ºC and 12ºC for all three chemokines, where the profiles at 4°C showed a clearer up-regulation at both 6 and 24 hours, while they at 12°C

dropped at 6 hours and increased again at 24 hours. This could indicate that the studied chemokines are particularly important for the initial phase of wound healing at low temperatures.

Other skin mucus proteins have been identified that can be relevant for future research on dietary modulation of skin, such as peptidyl-prolyl cis-trans isomerase (PPI-ase) and anterior gradient-2 homologue (AG-2). PPI-ase was separating all fish fed diets with functional additives from the fish fed control feed, which makes it an interesting candidate for further studies. PPI-ase belongs to a family of immunophilins, that similar to HSPs can facilitate protein folding, and plays different roles during stress (Bissoli *et al*. 2012). PPI-ases were originally recognised as protein receptors for an immunosuppressive drug, but novel roles may involve interaction with transcription factors, membrane transporters and other cellular proteins (Bissoli *et al.* 2012). Secreted forms of PPI-ases are inflammatory and chemotactic agents for monocytes, eosinophils and basophils (Galat 1993). The role of PPI-ase is not characterised in Atlantic salmon, but further investigation of the function of this protein and its role in mediating a possible immune response could be warranted.

AG-2 was down-regulated in the fish fed diet PX I in Paper IV, which had lower lice level than the control group. This protein is also known to be highly expressed in gills of Atlantic salmon suffering from AGD, and a link to recruitment of mucous cells of both skin and gills has been suggested (Morrison and Nowak 2008). AG-2 is described as an analogue of the *Xenopus* anterior gradient-2 (XAG-2) gene, which is responsible for differentiation of the dorsal ectoderm into the mucus gland (Aberger *et al*. 1998, Morrison *et al*. 2006). In AGD-affected salmon, AG-2 expression has been found to be upregulated in the lesions using microarray analyses, and immunohistochemical studies confirmed that AG-2 was highly expressed in cells within the lesions (Morrison *et al*. 2008, Morrison *et al*. 2006). A recent proteomic study of gill and skin mucus from AGD affected fish has also confirmed the high expression of this protein (Valdenegro-Vega *et al.* 2014.) No significant difference was seen in mucous cell area in Paper III, although

there was a small trend to more mucous cells in the control group with higher lice level. It is possible this could be related to the higher level AG-2 in this group. As earlier discussed mucous cells within the epidermis can be affected by external stressors, and a verification of AG-2 gene and protein expression in correlation with histological assessment of mucous cell changes would provide valuable knowledge to further understanding the mechanisms of mucous cell recruitment.

In conclusion, transcriptomic and proteomic methodologies have the potential to provide further insight into the protein response in mucosal surfaces of fish exposed to external stressors. The information from these three studies (Paper I, III and IV) provides a baseline for further research, for example validation studies on the effect of temperature, diets, sea lice or pathogenic disease on the epidermal mucus proteome.

5.4 Dietary impact on skin and mucus

As mentioned in chapter 5.1, the focus of Paper I was to document the chemical composition of skin tissue and mucus in Atlantic salmon and how this composition is affected by temperature. In the other three papers (II, III and IV), dietary supplementation with feed additives has been assessed both on skin composition and function. Several additives have been tested including nutrients such as vitamin C, E, Zn, immunostimulants (β-glucans, mannan oligosaccharides (MOS)) and a range of plant extracts or their chemically produced identical compounds. It is widely accepted that optimal nutrition leads to better health, and that nutritional imbalance can have a profound effect on growth, disease resistance and survival. The link between nutrition, dietary supplementation and health has been presented in books by Lim and Webster (2001) and Nakagawa *et al*. (2007), and more recently Kiron (2012) reviewed the research on immunonutrition and disease resistance in fish. Functional feeds are diets that can have positive effect on the health and/or growth promoting performance of the animals, by the supplementation of additional compounds beyond the basic nutritional

requirements (Tacchi *et al*. 2011). A range of materials are available for inclusion in functional feeds, mainly categorised as vitamins, minerals, pre- and probiotics, immunostimulants, nucleotides and plant extracts (Kiron 2012, Tacchi *et al*. 2011).

In terms of nutrient modulation of the skin, analyses of fish from Paper II showed that dietary supplementation with vitamin C, E and Zn resulted in a higher concentration of these nutrients in the skin (table 2; unpublished results). This suggests a potential role of these three nutrients to support skin function as indicated in Paper II, where fish fed diets with supplemented Zn showed an up-regulation of chemokines during the first 6 hours at 4°C after inflicting a wound. At 12°C, fish fed both diets with supplemented Zn showed less down-regulation than control after 6 hours, and stayed higher at 24 hours. The impact of Zn on chemokine expression was greater at low temperature (4ºC) which is in accordance with the results obtained in Paper I, as mentioned earlier. The first hours of wound healing are considered to be critical, and a quick response is important to cover the wound and restore homeostasis. The increase in chemokine expression in the Zn supplemented diets correlated with the dispersal of mucous cells in the epidermis two weeks post wounding. Although mucous cell differentiation is not fully understood, it is likely that Zn could play a role in the regulation. In addition to speeding up the re-epithelialisation process and modulating inflammation (Tenaud *et al*. 2000), Zn is also suggested to have a role in regulating epidermal cell mitosis in humans (Landsdown 2007). Knowing that Zn deficiency is associated with delayed wound healing and erosions of the fins and skin in fish (Fountoulaki *et al.* 2010, Ogino and Yang 1979), and also has an impact on immune response and disease resitance (Lim *et al*. 2001) it is necessary to secure sufficient supplementation of Zn in diets for salmon.

The Zn+ diet was also supplemented with vitamin C and E, β-glucans and a mixture of plant extracts (or their natural identical compounds). Vitamin C has been shown to improve wound healing in rainbow trout (Wahli *et al*. 2003), but the main effect was generally seen on fibrous tissue, including the repair of damaged dermal fibres, re-vascularisation and the re-establishment of normal dermal and muscle structure.

Table 2. Diet and skin levels (mg/kg) of Zn, vitamin C and E in Atlantic salmon fed three experimental diets at 4°C. Skin values are shown as averages ± sd. n = 10
(unpublished data related to Paper II)

	Control	**Zn**	**Zn+**
Zn (diet)	92	241	251
Zn (skin)	28 ± 6	42 ± 9	45 ± 6
Vit C (diet)	38	35	697
Vit C (skin)	29 ± 4	35 ± 7	55 ± 12
Vit E (diet)	215	218	697
Vit E (skin)	54 ± 6	54 ±7	57 ± 11

The lack of observed effect of vitamin C in Paper II could therefore be due to the focus epidermal healing. A prolonged study with focus on dermal parameters could potentially have revealed effects of the vitamin C supplementation. The content of vitamin E in the feed was well above requirement level in the control diet, and the increased inclusion in the feed had little effect on the concentration in the skin (table 2). The content in the control feed was probably sufficient to maintain antioxidant capacity and immune function (Hamre 2011, Lall 2000, NRC 2011). Additional stress or a disease challenge would be necessary to see the effect of different vitamin levels.

Immunostimulants have become an important part of fish health management programs, and among the most commonly used are the yeast sourced β-glucans. They have been demonstrated to enhance the resistance of a range of fish and crustacean species against bacterial and viral infections (Dalmo and Bøgwald 2008, Meena *et al*. 2013, Ringø *et al*. 2012). β-1.3/1.6-glucans derived from baker's yeast have been suggested to be the most potent immune-system enhancers (Ringø *et al*. 2010), and were included in the diets investigated in Papers II and IV. β-glucans have been shown to accelerate wound healing processes in mammals (Berdal *et al*. 2007, Koh and DiPietro 2013, Vetvicka and Vetvickova 2011), and to promote closure of wounds and induce local changes in cytokine expression in common carp (Przybylska-Diaz *et al*. 2013). β-glucans have also been shown to improve innate immune response in salmon by increasing the activity of

macrophages (Jørgensen and Robertsen 1995, Jørgensen *et al*. 1993). The role of macrophages wound healing is not fully understood, but they are known to promote inflammation and produce chemoattractants, including chemokines, which recruit additional cells to the wound site (Koh and DiPietro 2013). Although no effect of the added β-glucans could be demonstrated on the expression of the chemokines measured in Paper II, they could have an effect on other immune related genes not investigated in this study. A broader approach, e.g. a microarray screening of the expression of immune related genes, could provide more information on the effects of β-glucans on wound healing. With the current methodology described in Paper II, only supplementation of Zn could be shown to have impact on wound healing.

Sea lice are the main welfare issue and the most economically important ectoparasites on Atlantic salmon. Due to a reduction in the efficacy of medical treatments, new strategies for controlling the parasites are needed. In Paper III and IV, the addition of in-feed immunostimulants and plant extracts were tested in challenge trials with sea lice. The results of feeding functional ingredients to salmon on sea lice infestation have been discussed in detail in Paper IV. In brief, no effects were seen on lice numbers by feeding diets supplemented with β-glucans or mannan oligosaccharides (MOS). β-glucans have previously been shown to reduce lice burdens on salmon (Refstie *et al*. 2010, Ritchie 2000), and has also been found to improve resistance against another skin parasite, the ciliate *Ichthyophthirius multifilis,* in rainbow trout (Lauridsen *et al*. 2010). MOS has also been shown to reduce sea lice numbers in one trial with salmon (Sweetman *et al*. 2010), but failed to do so in a different trial (Refstie *et al*. 2010).

Although not significant, there was a trend of lower lice counts in all fish fed any of the functional diets in Paper III. These diets have been supplemented with different natural identical compounds of plant extracts, as shown in table 3 (unpublished data). In Paper IV, one of the diets containing natural identical compounds (Diet PX I) significantly lowered the average louse abundance by 20%, while diet PX II containing citral had no effect. There is a growing body of evidence indicating potential value of plant extracts as

control agents against ectoparasites, particularly lice, mites and ticks (Ellse and Wall 2013, Nerio *et al*. 2010). Plant essential oils (EO) are blends of plant metabolites, usually extracted through steam distillation, that have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications (Bakkali *et al*. 2008). Several EO, or their active components, have shown repellent activity against ectoparasites (reviewed by Anthony *et al*. 2005, Ellse and Wall 2013, Nerio *et al*. 2010). The repellent effect has mainly been studied in terrestrial farm animals, but tea tree oil and oregano oil have shown antiparasitic effects in three spined stickleback (*Gasterosteus aculeatus*) and sea bass (*Dicentrarchus labrax*) (Yiagnisis *et al*. 2009, Steverding *et al*. 2005). There is also evidence that synergies between the metabolites of EO may result in higher bioactivity than the pure components (Hummelbrunner and Isman 2001). This could apply to diet PX I, which contained a combination of natural identical compounds of EO. This has been suggested to be due to the fact that plants usually present defenses as a suite of compounds, not as individual metabolites (Nerio *et al*. 2010).

Table 3. Functional ingredients tested in Paper III and IV

Diet	**Functional ingredient**
Paper III	
A (Control)	-
B	Carvacrol
C	Cinnamic aldehyde
D	Eugenol
E	Tea tree oil
Paper IV	
PX I	Mix of plant extract*
PX II	Citral

*A commercial, proprietary mix of natural identical compounds (Lucta, Barcelona, Spain)

The effects of both plant extracts and immunostimulants on disease resistance are known to depend on the nature of the tested substance, the inclusion levels in the feed and the duration of administration (Bricknell and Dalmo 2005, Burrells *et al*. 2001a, Dalmo and Bøgwald 2008), as well as the severity of the challenge, in this case the number of parasites per fish. A reduction of 20% lice attachment as seen with the PX I diet is a valuable contribution to the control of the parasite, but more studies are required on the sources, doses and methods of action of these additives.

6. Conclusions

The current work has demonstrated that exogenous factors can change the composition, structure and functionality of Atlantic salmon skin. Three such factors have been employed: temperature change, sea lice infection and wound infliction. From this thesis the following main conclusions can be drawn:

- The methodologies used in the thesis have proven to be useful in assessing changes in skin and mucus. Quantitative histology using digital image analysis, proteomics of mucus and transcriptomics of skin all demonstrated to be valuable tools in defining differences between groups exposed to distinct treatments.

- Chemical composition in Atlantic salmon skin was affected by temperature. Increasing temperature resulted in higher level of protein and collagen related amino acids, while moisture level was reduced. A higher degree of unsaturated fatty acids was observed in the skin at low temperature, which could be related to the maintenance of membrane fluidity. Increasing the dietary levels of minerals (Zinc) and vitamin C resulted in an increase in their concentration in the skin.

- The epidermis was significantly thinner at higher temperature, with greater mucous cell coverage. The epidermis was thicker in the dorsal than cranial area. Sea lice infection had no impact on these parameters. The skin morphology during wound healing was affected by time, temperature and diet. Elevated temperature and dietary zinc advanced wound healing as assessed by mucous cell position.

- Temperature had an effect on the skin transcriptome. A pre-activation of the skin mucosal immunity was observed at low temperature. Chemokine gene expression was affected by temperature, being higher at low temperature both in normal skin and during wound healing. Up-regulation of a number of heat shock proteins correlating

with a decrease in epidermal thickness suggested a stress response in the skin at high temperature.

- Proteomics is a valuable non-invasive method to assess changes in mucus. A large number of proteins were identified, several of which responded to sea lice infection and dietary modulation (chemokines, HSPs, AG-2, calmodulin and peptidyl-prolyl cis-trans isomerase). After validation, these proteins could be used as potential biomarkers to assess skin functionality.

- Diets supplemented with plant extracts reduced sea lice infection even under high challenge pressure, and could be part of an integrated pest management strategy.

7. Future perspectives

There is a general agreement that the skin of fish is an essential organ as a first line mechanism of defence, but detailed information on salmon skin and its mucus layer is still scarce and requires further investigation beyond this thesis.

Temperatures outside the optima for the species have been shown to induce significant changes in the skin in terms of composition, structure and functionality. However, their potential impact on skin pathologies at suboptimal temperatures is not yet clearly understood. The physio-pathological significance of the alterations observed (e.g. composition, epidermal thickness, mucous cell coverage and expression of HSPs) and resulting impact on the incidence of skin condition requires additional research.

Image analysis through quantitative histology was in the present work limited to a few skin parameters, mainly epidermal thickness and mucous cell coverage. Although they were relevant to assess the function of skin as a physical barrier against external agents, these two parameters provided limited information on other skin functions. The combination of quantitative histology with differential staining techniques, including immunohistochemical methods to identify specific cell populations or tissue structures, can contribute to a better understanding of skin condition.

To be able to study more subtle changes in the complex phases of wound healing, a less invasive method to inflict wounds should be developed. The time course of sampling could also be optimised by increasing the sampling frequency in the early phase, and extending the duration of the study until the structure of the epidermis is completely reorganised. Another approach could be to establish a salmon skin cell line or a skin organ culture model. This would reduce the number of experimental animals, and in addition to study wound healing and infections, such a model could be useful for a wide range of research fields.

Over the last years there has been an increased interest in mucosal immunity, and more documentation has become available in this area. Fish skin mucus contains a range of components that can have an effect against a variety of pathogens. Changing the mucosal immunity through diet has proven to be possible, and more knowledge could help reducing the need for medication and chemical treatment in fish farming. A focus area could be the use of genetic and proteomic methods to monitor the impacts of stress and disease or the effect of dietary modulation. While the changes observed in the mucus proteome offer great potential, utilising the information from the fully annotated salmon genome will allow more resolution to future analysis. Antimicrobial peptides or other components from fish mucus may even be interesting for treating human pathogens, and the field of mucosal immunity is likely to grow over the next years. A deeper understanding of the skin component of the mucosal immunity of fish will also help the future development of vaccines and other immunotherapeutic, and thus contribute to mitigate the lice situation on all farming regions affected by the parasite.

Future studies can further enhance the understanding of functional diets to support skin health. These could become intrinsic components of integrated pest management strategies and reduce reliance on chemotherapeutants.

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