Aquaculture relevant stressors and their impacts on skin and wound healing in post-smolt Atlantic salmon (*Salmo salar L.*)

Lene Rydal Sveen

Avhandling for graden philosophiae doctor (ph.d.)
Universitetet i Bergen
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Navn: Lene Rydal Sveen

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Scientific environment

The work in this PhD thesis was performed at Nofima in the Department of Aquaculture, the University of Bergen at the Department of Biological Sciences, and UNI Research. Experimental work was done at the Industrial and Aquatic Laboratory (ILAB) located at the University of Bergen and at Nofimas Centre for Recirculation in Aquaculture (NCRA) at Sunndalsøra.

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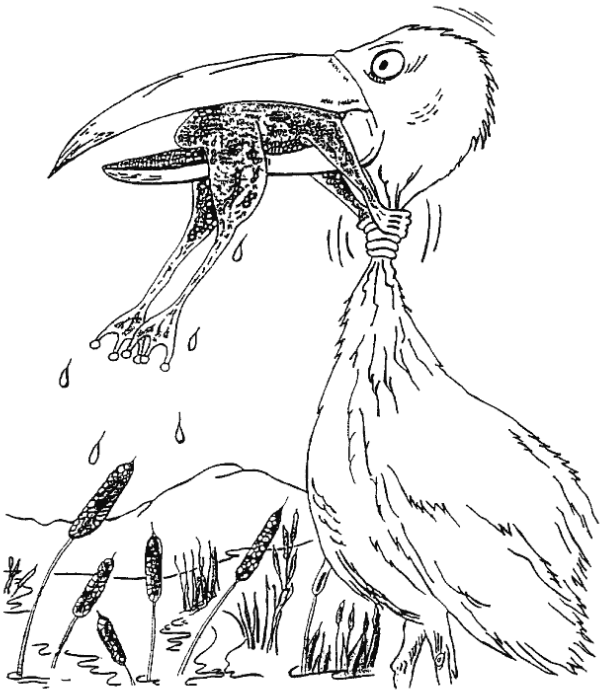
A special thanks to my wonderful little family. Rikke, you wake up every morning with a big smile on your face and Fabian you have to listen to complains, giving advices, and reading manuscript drafts, still not complaining that much! Also a great thanks to my mom which helped a lot babysitting (and having fun) with the little princes. I also carry the spirit of Tom in me, the main message being never ever give up!



Never ever give up !

Abstract

The work described in this thesis focuses on the response mechanisms in the skin of post-smolt Atlantic salmon (*Salmo salar* L.) under the following challenging conditions:

i. The effect of high biomass and low specific water flow on skin integrity (paper I)
ii. The effect of high biomass followed by acute challenge test on mucin transcription in skin (paper II)
iii. General wound healing mechanisms in Atlantic salmon skin (paper III) and the effect of high biomass on wound healing (paper IV)
iv. The effect of handling stress on mucin transcription (paper II)

High biomass is predicted to provide greater profitability in the production of fish in closed systems. Similarly, low water flow can reduce costs associated with water treatment and water supply. In paper I, these hypotheses were challenged. Five fish densities (25, 50, 75, 100 and 125 kg/m^3) and four levels of specific water flow (0.2, 0.3, 0.4 and 0.5 l /kg fish/min) were established to detect effects on salmon skin. After eight weeks, both high biomass (≥ 100 kg/m^3) and low specific water flow, (0.2-0.3 l /kg/min) led to activated stress and immune responses in the skin of post-smolt, including increased transcription of mucin-like genes.

The main constituency of the mucus layer are big gel-forming proteins called mucins. In paper II, seven gel-forming mucins, including two *mucin2* and five *mucin5* variants, were identified based on a computational pipeline consisting of annotation, transcription, domain structure and phylogenetic analysis. *Mucin2* was predominantly expressed in the intestine, while the *mucin5* family was expressed in many organs, including skin and gills. In order to investigate transcriptional regulation of mucins during stress conditions, two controlled experiments were conducted. In the first experiment, handling stress induced mucin transcription in the gill, while transcription decreased in the skin and intestine. In the second experiment, long term intensive

rearing conditions interrupted by additional acute challenge test led to increased transcription of mucin genes in the skin at one, seven and fourteen days post-challenge.

Farmed fish are particularly vulnerable to skin damage which may occur after handling, confinement, infections and more. The same procedures may trigger stress responses in the fish. Thus, the goal of paper III was to describe the general cutaneous wound healing mechanisms in post-smolts while paper IV focused on the effects of chronic stress on wound healing. Two experimental groups were created where low fish density was used as control ($\bar{x}$ = 14 kg/m^3) and high fish density ($\bar{x}$ = 126 kg/m^3) as chronic stress factor. Deep cutaneous wounds were inflicted on the flank of the fish by using a 5mm punch biopsy tool and the following healing process was documented with seven sampling points over a period of 53 days. The results showed that the healing process was dominated by an early acute inflammatory phase and a later regenerative stage with connective tissue formation and wound contraction. Both the histological analyses and the transcriptional responses fully supported these findings. Stress changed several steps in the healing process, including re-epithelialization, the mucus response, scale mineralization, pigmentation, formation of fibrous tissue and wound contraction, as demonstrated by histology and picture analysis. The transcriptional analyses showed that the inflammatory response was enhanced by chronic stress, while several genes involved in tissue repair were downregulated. These alterations may be used as key check points in future studies when evaluating the effect of a given treatment on cutaneous wound healing.

In conclusion, the challenging conditions applied in this thesis in general enhanced the inflammatory reactions or altered the mucus response in the skin of post-smolt Atlantic salmon. Molecular analyses on intact skin and wound samples can thus be used to detect adverse effects of the aquatic rearing environment, including effects that cannot be detected by traditional welfare analyses.

Sammendrag

Arbeidet i denne avhandlingen omfatter fire hovedområder, alle designet for å detektere ulike responsmekanismer i huden til Atlantisk lakse (*Salmo salar*):

i. Effekten av høy biomasse og lavt vannforbruk (artikkel I)
ii. Effekten av høy biomasse og akutt stress på mucintranskripsjon i skinn (artikkel II)
iii. Sårhelingsmekanismer i lakseskinn (artikkel III) og effekten av kronisk stress på sårheling (artikkel IV)
iv. Effekten av håndteringsstress på transkripsjon av mucingener (artikkel II)

Høy biomasse er ønskelig for å øke lønnsomheten ved produksjon av fisk i lukkede systemer. På samme måte kan lav vanngjennomstrømning redusere kostnader knyttet til vannbehandling og vannforsyning. Fem ulike tettheter (25, 50, 75, 100 og 125 kg/m^3) og fire nivåer med spesifikk vanngjennomstrømning (0.2, 0.3, 0.4 og 0.5 l/kg fisk/min) ble brukt for å vurdere effekter av tetthet på laksens hud (artikkel I). Etter åtte uker førte både høy biomasse (≥100 kg/m^3) og lav vanngjennomstrømning (0.2-0.3 l/kg/min) til aktiverte stress og immunresponser i huden til post-smolt, deriblant økt transkripsjon av mucin-liknende gener.

Mucinene danner slimlag som er med på å beskytte epitelcellelagene i barrierevev i hud, gjeller og tarm. Basert på annotasjon, transkripsjon, domenestruktur og fylogeni, ble syv gel-dannende muciner identifisert: to *mucin2* og fem *mucin5* varianter. *Mucin2* ble i hovedsak uttrykt i tarmsystemet, mens *mucin5*-familien var utrykt i mange organer, deriblant hud og gjelle. Videre ble det viste at håndteringsstress økte mucintransskripsjon i gjelle, mens transkripsjonen ble redusert i hud og tarm. Høy biomasse fulgt av 30 min med akutt trenging, førte til økt transkripsjon av mucingener i huden.

Oppdrettsfisk er utsatt for vevskader som oppstår som følge av håndtering, behandling, infeksjoner med mer. Slike skader på huden representerer en viktig infeksjonsvei for

patogener. Oppdrettsfisk er også utsatt for stressorer som potensielt kan skade fiskens helse og velferd. For å lære mer om generelle sårhelingsmekanismer i laks samt effekten av stress på sårheling ble det etablert to grupper: lav fisketetthet ble brukt som kontroll ($\bar{x}$ = 14kg/m^3) og høy fisketetthet ($\bar{x}$ = 126 kg/m^3) som stressfaktor. En 5 mm hudbiopsi ble stanset ut og sårhelingsprosessen ble deretter fulgt over en periode på to måneder. Sårhelingsprosessen i både kontroll og stresset fisk var dominert av en tidlig akutt inflammatorisk fase og en senere fase med bindevevsdannelse og sårkontraksjon. Både de histologiske analysene og transkripsjonsanalysene støttet i sin helhet opp om disse resultatene. Kronisk stress endret flere trinn i helbredelsesprosessen, deriblant re-epitelialiseringsprosessen, mukusresponsen, mineralisering av skjell, pigmentering, dannelse av bindevev og sårkontraksjonen. Transkripsjonsanalysene viste videre at inflammasjonsresponsen i all hovedsak ble forsterket av kronisk stress, mens flere gener involvert i vevsreparasjon ble nedregulert. Det antas at de ovennevnte endringene kan benyttes i fremtidige studier for å evaluere effekten av en gitt behandling på sårheling i huden hos laks.

Kort oppsummert viser resultatene i denne avhandlingen at ulike stressorer øker inflammatoriske reaksjoner og endrer slimresponsen i huden til post-smolt. Molekylære analyser på intakt hud og sårprøver kan dermed brukes til å oppdage negative effekter av oppdrettsmiljøet, inkludert effekter som ikke kan oppdages ved tradisjonelle velferdsanalyser.

List of publications

Paper I

Lene Rydal Sveen, Gerrit Timmerhaus, Jacob Seilø Torgersen, Elisabeth Ytteborg, Sven Martin Jørgensen, Sigurd Handeland, Sigurd O. Stefansson, Tom Ole Nilsen, Sara Calabrese, Lars Ebbesson, Bendik Fyhn Terjesen, Harald Takle. Impact of fish density and specific water flow on skin properties in Atlantic salmon (*Salmo salar L.*) post-smolts. Aquaculture 464 (2016) 629–637

Paper II

Lene Rydal Sveen, Fabian Thomas Grammes, Elisabeth Ytteborg, Harald Takle, Sven Martin Jørgensen. Genome-wide analysis of Atlantic salmon (*Salmo salar*) mucin genes and their role as biomarkers. PLoS ONE 12(12): e0189103.

Paper III

Lene Rydal Sveen, Gerrit Timmerhaus, Aleksei Krasnov, Harald Takle, Sigurd O. Handeland, Elisabeth Ytteborg. Wound healing in post-smolt Atlantic salmon (*Salmo salar*). (*Submitted manuscript, Nature Scientific reports*)

Paper IV

Lene Rydal Sveen, Gerrit Timmerhaus, Aleksei Krasnov, Harald Takle, Sigurd O. Handeland, Sigurd O. Stefansson, Elisabeth Ytteborg. Chronic stress delays wound healing in Atlantic salmon (*Salmo salar*). (*Submitted manuscript, Nature Scientific reports*)

Contents

1. Introduction

1.1 New technologies in post-smolt production

There is an ongoing change in the practice of production of Atlantic salmon (*Salmo salar*) post-smolts in the Norwegian aquaculture sector. Several commercial companies are developing and investing in equipment for post-smolt production in semi-closed containment systems (S-CCS) at sea or in closed-containment systems (CCS) in land-based facilities (Iversen et al., 2013). As the names imply, S-CCS and CCS offer a semi-closed or fully closed interface between the fish and the surrounding environment. Traditionally, smolt are reared in land based freshwater facilities and transferred to open sea cages at 60-100 grams where they grow until harvest. Once at sea, elevated mortalities occur in connection with poor smolt quality, outbreak of diseases and handling operations such as sea-lice treatments [1]. Rearing post-smolt in S-CCS or CCS will reduce the production time in open sea cages. As a result, production in these systems has been highlighted as a key solving important challenges, such as mortalities in sea, sea-lice pressure and as a way to eliminate escapees [2].

In 2014, it was generally assumed that intensification of S-CCS and CCS was required to make these systems profitable [2, 3]. Reduced specific water flow (reduced water treatment costs) and increased fish densities (higher production per volume) are two of the main factors that may increase the profitability of post-smolt production in S-CCS and CCS [4, 5]. However, since 2014 the production costs for open sea-cages have increased dramatically, mainly associated with costs related to treatment of salmon lice (*Lepeophtheirus salmonis*) [6]. As a result, estimated production costs in open sea-cages and S-CCS and CCS are converging [2, 7]. Even though the cost gap has decreased, increased intensification will still lead to increased economic viability [2, 8]. Therefore, the question is how to proceed with a sustainable intensification of post-smolt production, while at the same time securing the welfare of the animal.

1.2 Skin lessions and stressors, a combined threat to animal welfare.

According to the Norwegian animal welfare act, farmers must ensure that the animals are kept in an environment that provides good welfare based on the requirement of the species and individual needs, including stimulating activities and space for movement, rest and other natural behavior [9]. The legislation regarding animal welfare takes into account the three main philosophical concepts concerning animal welfare. The function based approach consider animals that spend little time and energy coping with the environment to have good welfare [10]. The feeling based approach, takes the animal feelings into account and it follows that animals which are feeling well also have good welfare [11]. The behavior-based approach consider the ability of the animal to express natural behaviors and live natural lives [11, 12]. The function-based approach is most commonly used in welfare assessments. Fish performance can be measured on group level such as feed intake, appetite, growth pattern, behavior, mortality or on the individual level such as deformities, skin lesions, cataract, blood parameters and more [13]. These measurements are often done in combination with factors that describe the rearing environment [13]. In general, the rearing environment is adapted to the requirements of the fish (feeding, light regimes, water flow etc.), but there are still several factors which may trigger stress responses in the animal. All fish are exposed to stressful handling procedures such as vaccination, handling and pumping [14]. In addition, intensification of post-smolt production in S-CCS and CCS may result in unfavorable rearing conditions triggering stress responses in the fish [4, 5]. If the stress response becomes severe or long lasting it changes the metabolic state of the animal and increases the susceptibility of diseases [15-17].

The stress response itself consists of a complex suite of both hormonal, neural and physiological modifications. Adrenaline and glucocorticoids such as cortisol are primary stress hormones that regulate numerous physiological processes in an effort to maintain homeostasis [18, 19]. The secondary responses are triggered by the changes in circulating levels of cortisol and adrenaline, such as mobilization of energy,

increased heart stroke volume, and blood flow to gills and muscle [20]. When the stress responses are severe or long lasting, a series of tertiary effects become apparent. This include changes in immune function, disease resistance, growth and reproduction. As fish reared in aquaculture facilities have little ability to escape from their environment, sub optimal rearing conditions may trigger chronic stress responses resulting in an energy drain which leads to trade-offs with other energy-demanding processes such as the functioning of the primary epithelial barriers (gut, skin, gills), the immune system, growth and reproduction [16]. It has been shown that skin as the first barrier tissue in Atlantic salmon respond to changes in the environment, such as temperature, stress, nutrition and rearing conditions [21-26]. Thus, environmental effects on skin appear fast and skin analyses may be used to detect suboptimal rearing conditions. As it is easy and cheap to visually inspect the outer surface of the fish, several features of the skin such as scale loss, bleeding, skin lesions, ulcers and fin erosions are commonly used in welfare assessments [13].

1.3 Salmon skin

The skin of Atlantic salmon is a coherent and dense barrier that protects the interior of the fish against the outer environment. The skin carries out multiple functions such as communication, protection, sensory perception and locomotion [27]. The skin is a multilayered organ, consisting of four different layers with distinct properties (Fig. 1). The mucus layer is a viscoelastic gel build from mucus proteins, which lubricates and protects the epithelial surface. The epidermis consists of live cells that cover and seal the surface and contribute to the mucus layer and innate immunity, while the dermis provides strength and elasticity to the skin. The hypodermis contribute with isolation and pigmentation.

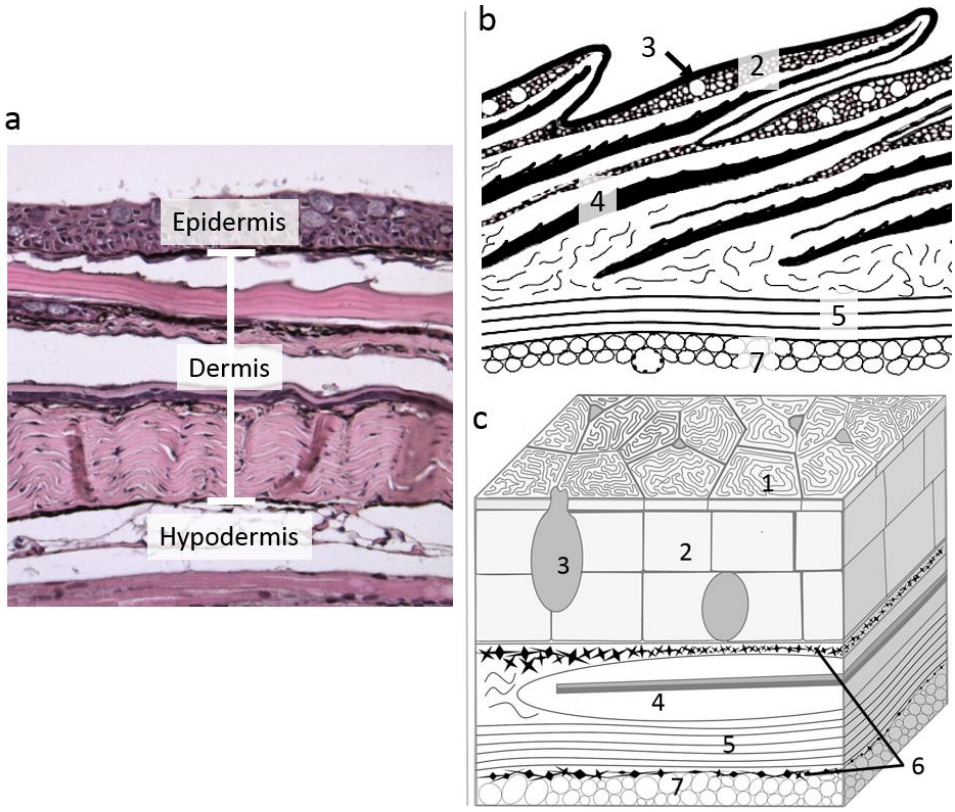


Figure 1. Atlantic salmon skin. a. 5 μm skin section stained with hematoxylin and eosin. **b**. Schematic drawing of fish skin with overlapping scales **c.** Schematic drawing of the cellular layers of Atlantic salmon skin. The keratocytes in epidermis are enlarged to highlight the difference in morphology between the flat keratocytes and the intermediate keratocytes. 1. Flat keratocytes, 2. Keratocytes, 3. Mucous cell, 4. Scale and scale pocket 5. Dense connective tissue 6. Pigment cells 7. Hypodermis. Figure is inspired by Elliot [27].

Epidermis

The epidermis is the first cellular layer of the skin. The thickness of epidermis will vary from just a few layers of cells to more than 20, depending on body site and age [24, 28]. In Atlantic salmon there are two cell types in the epidermis, keratocytes and mucous cells [29]. The keratocytes are the main cell type found in epidermis. In the literature, this cell type is also referred to as; keratinocytes, epidermal cell, epithelial cell, malpighian cell, principal cell, common cell and filament containing cell. One of the main features of the keratocytes are the structural changes that they undergo depending on where they are located in the epidermis (Fig. 2).

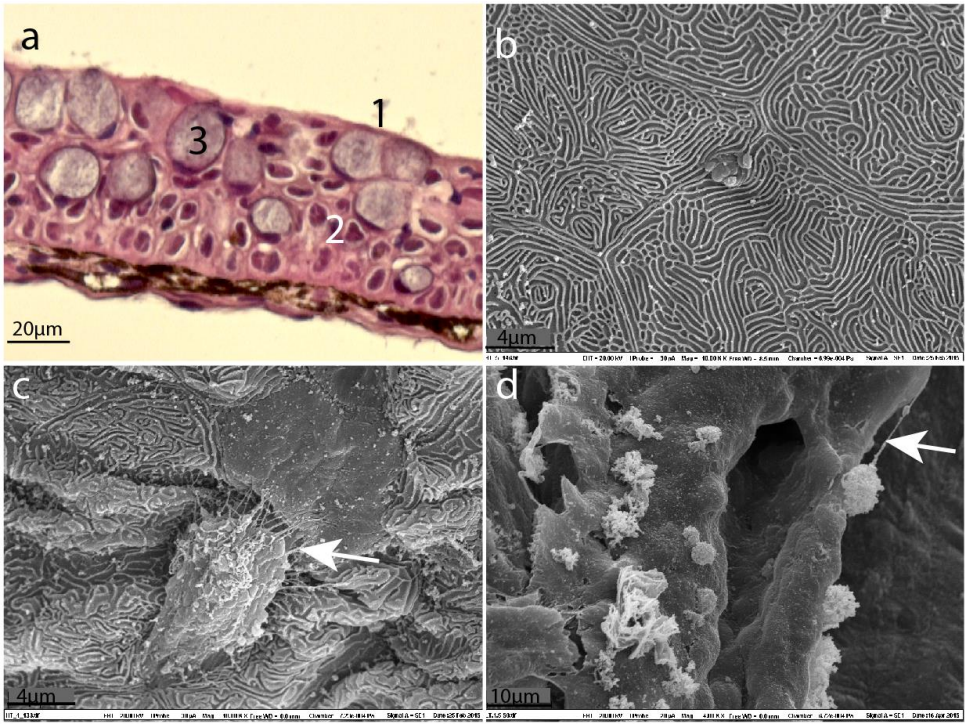


Figure 2. Shapes of keratocytes in Atlantic salmon skin a. Epidermis**,** 5 µm section stained with hematoxylin and eosin. **b.** Scanning electron microcopy (SEM) of the flat keratocytes with their characteristic microridges. **c** and **d.** Migrating keratocytes on wounded surface**.** Symbols: 1. Flat keratocytes, 2. Keratocytes, 3. Mucous cell. Arrow points to keratocyte with pseudopod.

At the epithelial surface, the keratocytes are flat, and they often have a pentagonal shape with elevated actin bands (microridges) at the surface (Fig. 2b). These elevated actin bands are suggested to assist in holding the mucus layer on to the cell surface [30]. The keratocytes which are found in the intermediate layers of the epidermis have a round shape, while the keratocytes that are attached to the basement membrane are square or cubical shaped [27]. Basal keratocytes are linked to the basement membrane with hemidesmosomes providing strength to the epidermis. In addition, adjacent keratocytes are linked to each other with components of the cytoskeleton (tonofilaments) attached to desmosomal plaques that join adjacent cells and enables the epidermis to respond to mechanical stress [27]. Further, there is increasing evidence pointing towards immune-cell like properties in the keratocytes. Keratocytes in culture

can internalize particular matter such as latex beads [31] and this property may aid in clearing wounds from pathogens [32-34]. These characteristics suggest that keratocytes are important contributors to the fish innate immune responses by providing active protection of the epidermal surface.

Mucous cells

The second cell type found in the epidermis are the mucous cells. Evidence suggests that keratocytes may differentiate into mucous producing cells [27], however this is still debated. As the mucous cells mature, they increase in size and the nucleus and organelles become displayed basally. Upon reaching the surface, the mucous cells emerge between adjacent epithelial cells and the mucus is released (Fig. 2b). The number of mucous cells vary with body site. The number of mucous cells is highest in the dorsal area and lower in caudal and anterior regions [23, 35, 36]. Natural variations such as sex and life stage may alter the number of mucous cells [37]. As an example the number of mucous cells decreases with 50% upon smoltification [38]. Toxic and irritating substances can stimulate mucus secretion and increase the thickness of the mucus layer [39-43], as can pathogens [44, 45], handling stress [46] and different rearing regimes [24].

The mucus gel and mucins

The main role of the mucus gel is to protect and lubricates the epithelial surface. In addition many biologically active molecules are dissolved in the mucus matrix such as enzymes, peptides and immunoglobulins which directly attack microbes and parasites and contribute to the protection of the underlying epithelia [37]. The mucus matrix may also act as a physical trap by immobilizing pathogens. The subsequent "washing of old mucus" is believed to be the main mechanisms that prevents colonization of pathogens [47]. Fish mucus is also known to be involved in many biological functions such as respiration, reproduction, parental feeding, nest building and more [37, 48, 49].

The main constituency of the mucus gel are large glycoproteins known as mucins. In humans and higher vertebrates more than twenty different mucins have so far been

identified [50]. The mucins are either synthesized and secreted by specialized cells known as mucous cells or presented at the apical surface of epithelial cells as transmembrane proteins. This separates the mucins into two functional classes: secreted mucins and membrane-bound mucins (Fig. 3). The secreted mucins are further divided into gel forming mucins and soluble mucins.

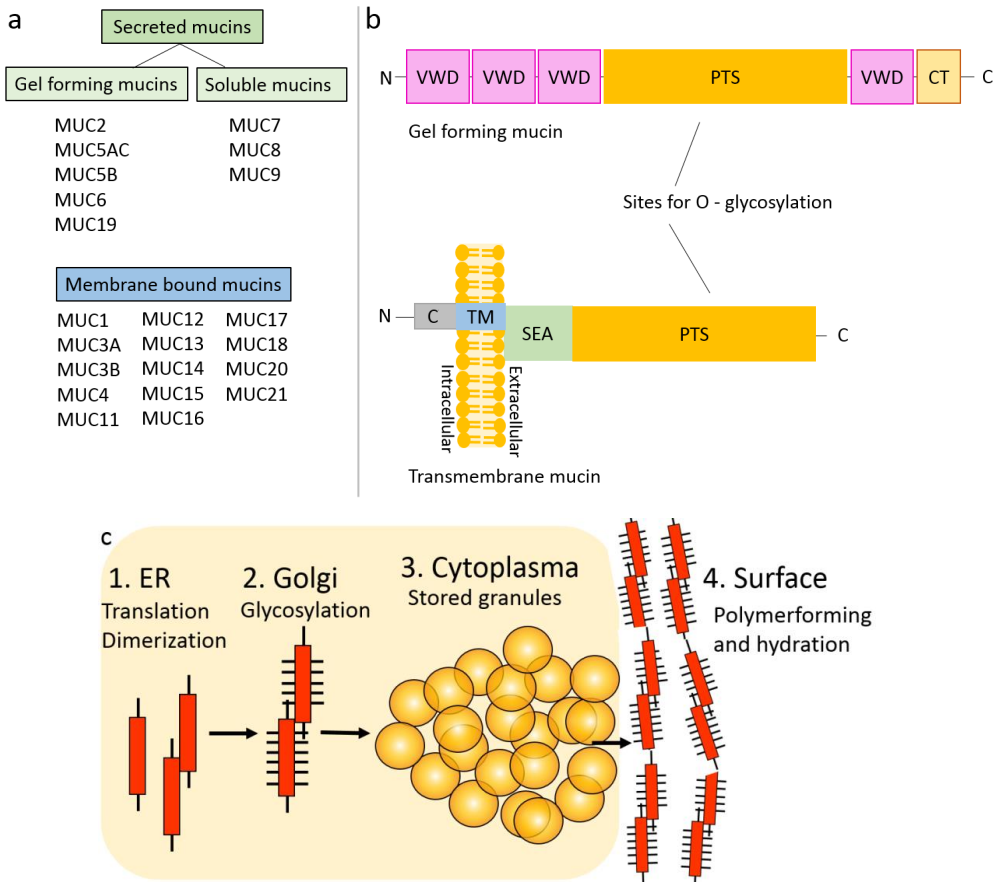


Figure 3. Classification and mucin structure a. Classification of mucin proteins **b.** Schematic presentation of secreted gel-forming mucins and membrane bound mucins. Cysteine knot (CT) domain, cytoplasmic (C) domain, PTS domain, sperm protein enterokinase and agrin (SEA) domain, transmembrane (TM) domain, von Willebrand Factor D (VWD) domain. **c.** Secreted gel forming mucins dimerize via their C-terminal domains, they are further O-glycosylated, condensed and packed into granules for secretion. Following secretion, the granules hydrate and swell and the gel mucus is formed. The figure is inspired by McGuckin et al. 2011 and Zaretsky et al. 2013 [50, 51].

The gel forming mucins are able to create a tangled web of linear polymers following N‑terminal dimerization (Fig. 3 c), or a complex molecular network following N‑terminal trimerization [50]. Upon secretion these networks of mucin proteins hydrate and swell in a process where mucus is formed. The soluble mucins are also secreted, but they do not have gel-forming properties. All mucins, regardless of being secreted or membrane-bound, have a central region rich in the amino acids proline, threonine and serine. In the literature this domain is referred to as the PTS domain, or variable number tandem repeats domain (VNTR) [52]. The PTS-domain is heavily glycosylated and the carbohydrates may account for as much as 70-90% of the mucin mass [53, 54]. Glycans that are attached to the PTS domain through serine and threonine residues are referred to as O-glycans and the enzymes involved in glycosylation are referred to as glycosyl transferases [51, 55].

The N and C-terminal domains of the gel-forming and membrane bound mucins have major structural differences. The N-terminal domain of gel forming mucins always have three von Willebrand Factor D (VWD) domains, cysteine rich (C8) and trypsin inhibitor like cysteine rich (TIL) domains[51]. The C-terminal has one VWD domain, C8 and TIL domains and a cysteine knot (CT) domain. The VWD and CT domains contribute to oligomerization of mucin proteins through disulfide bond formation [56], which gives the mucins their gel forming properties [50]. Thus, VW factor domains are lacking in soluble mucins [57]. In humans, MUCIN2, MUCIN5AC and MUCIN5B all have the domain architecture (VWD-C8-TIL)-(VWD-C8-TIL)-(VWD-C8-TIL)-PTS-(VWD-C8-TIL) [58], a similar domain structure have been found in Atlantic salmon gel-forming mucins [59]. Domains that are unique for transmembrane mucins are sperm protein enterokinase and agrin domain, transmembrane domain and cytoplasmic domain (Fig. 3b).

From studies in mammals, it is clear that a wide range of stimuli can trigger increased mucin transcription and expression, including bacteria, viruses, cytokines, chemokines, growth factors, mechanical stress, reactive oxygen species, epigenetic regulations and more, as reviewed by several authors [51, 60-63] (Fig. 4). Most external stimuli activate

a signal cascade that in turn activates transcription factors that bind to the mucin promotor regions [51, 62]. Several signaling pathways have been demonstrated to be involved, most frequently the MAPK/ERK pathway, but also the cytokine JAK-STAT and TGFβ-SMAD pathway [64]. Increased mucin expression can also be acquired by the cells through increasing stability of mucin mRNA. Neutrophil elastase, TNFα and IL-8 may up-regulate mucin expression by increasing the mRNA stability [65-67]. In contrast to the agents that up-regulate mucin expression, glucocorticoids have the opposite effect by inhibiting mucin transcription [68-70].

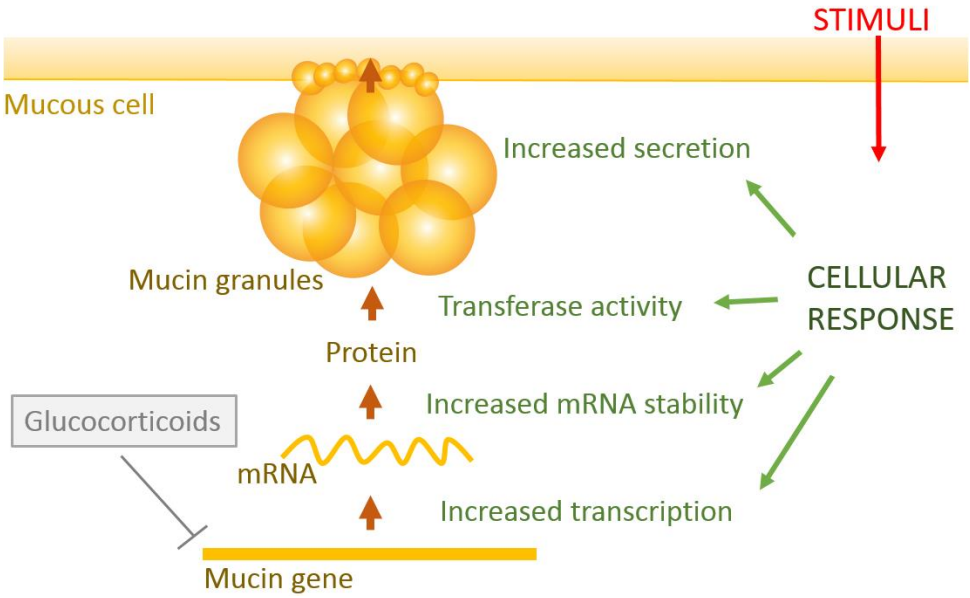


Figure 4. Factors that can affect mucus production. External stimuli can affect mucus production on the transcriptional level, by increasing mRNA stability, altering transferase activity and glycosylation pattern and changing the secretion rate of the mucin granules. Glucocorticoids inhibit mucin transcription. The figure is based on information in Zaretsky & Wreschner 2013 [51].

Most of the few published articles regarding mucin transcription in fish focus on pathogen host interaction in the gastrointestinal (GI) tract, reviewed by Quintana-Hayashi et al. [55]. As an example, mucin gene transcription in the GI tract of gilthead seabream (*Sparus aurata*) responded to both diet and infection [71]. Similarly, mucin transcription was altered in the gastrointestinal-tract of channel fish (*Ictalurus punctatus*) upon bacterial infection with *Edwardsiella ictaluri* [72].

Dermis

The dermis is the part of the skin located between epidermis and a thin cellular layer called hypodermis (Fig. 3 and 5). The dermis is again subdivided into two parts, stratum laxum and stratum compactum, meaning the loose and the compact part of the dermis. The first layer of the dermis consists of loose connective tissue and scales. In this layer a variety of cells and structures can be found such as blood vessels, nerve fibers and a diversity of pigment cells such as melanophores, iridohores and lipophores [27, 28]. The pigments cells gives the skin its distinct colors, while at the same time aid in camouflaging and communication [28]. The roles of the pigment cells are not fully understood, and it is suggested that these cells are also associated with wound healing and inflammation in salmonids [73-75].

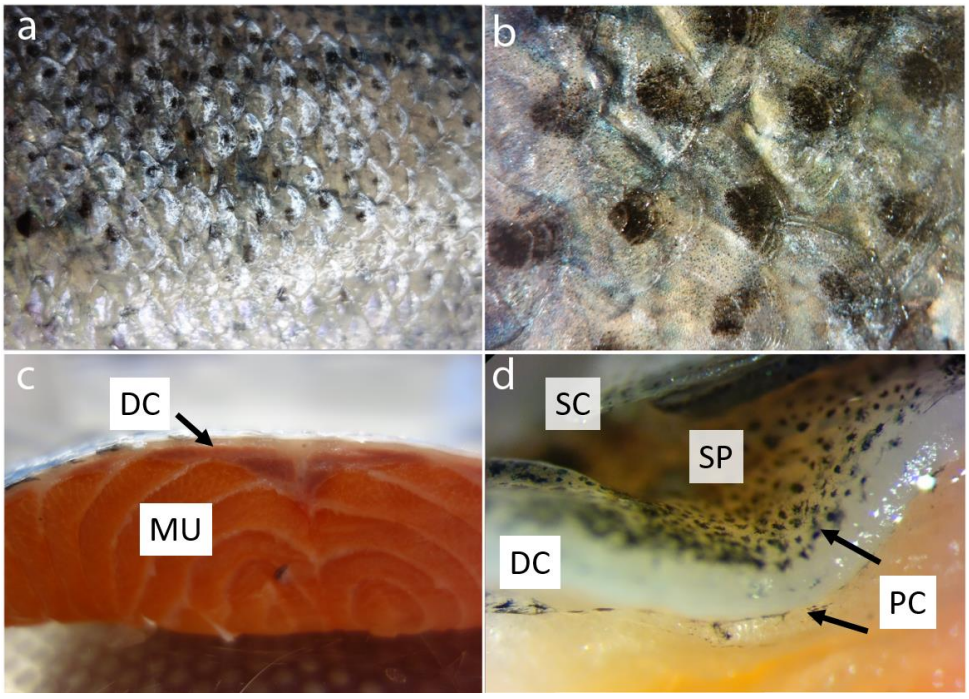


Figure 5. Macrostructures in the dermis a. Photo of fish skin with overlapping scales **b.** Overlapping scales (stereoscope 4X) **c.** Photo of fish skin and the underlying muscle **d.** Macrostructures of the different layers of dermis. Note that there are two layers of pigment cells above and below the dense connective tissue (stereoscope 40X). Symbols: dense connective tissue (DC), scale (SC), scale pocket (SP), pigment cells (PC).

The scales are small boney plates located in scale pockets in the dermis (Fig. 5). The main function of the scales is to provide physical strength to the skin. The scales in salmon have an elliptical shape (cycloid scales), with an upper mineralized layer of hydroxyapatite ($Ca_5(PO_4)_3(OH)$) and a lower fibrous layer of un-mineralized matrix and collagen fibers [76-79]. Thus, the scales may also act as a reservoir for calcium and phosphorus [80, 81]. The last layer of dermis is a continuous layer of dense connective tissue (Fig. 1 and Fig. 5c, d). This layer provides strength, tension and flexibility to the skin. The dense collagen fibrils, mainly consistent of collagen type 1 synthesized by fibroblasts [82].

Hypodermis

The dermis is separated from the underlying skeletal muscle by the hypodermis (Fig. 3). This is a well-vascularized layer that consists of loose connective tissue, blood vessels, nerves, adipocytes and pigment cells [27]. The upper layer of the hypodermis consist of pigment cells, whereas the major part of the hypodermis is comprised of adipose tissue.

1.4 Wound healing

If the case of wounding, a well-coordinated sequence of reactions is activated aiming to limit the injury, restore hemostasis, protect the damaged area from infection and further regain the architecture of the tissue and possible restore tissue function. In fish and other vertebrate the wound healing cascade consist the following overlapping phases; re-epithelialization, inflammation, granulation tissue formation and tissue remodeling (Fig. 6) [83]. The steps of cutaneous wound healing in fish are comparable to cutaneous wound healing in mammals, however in fish re-epithelialization precedes inflammation and blood clot formation does not occur [83].

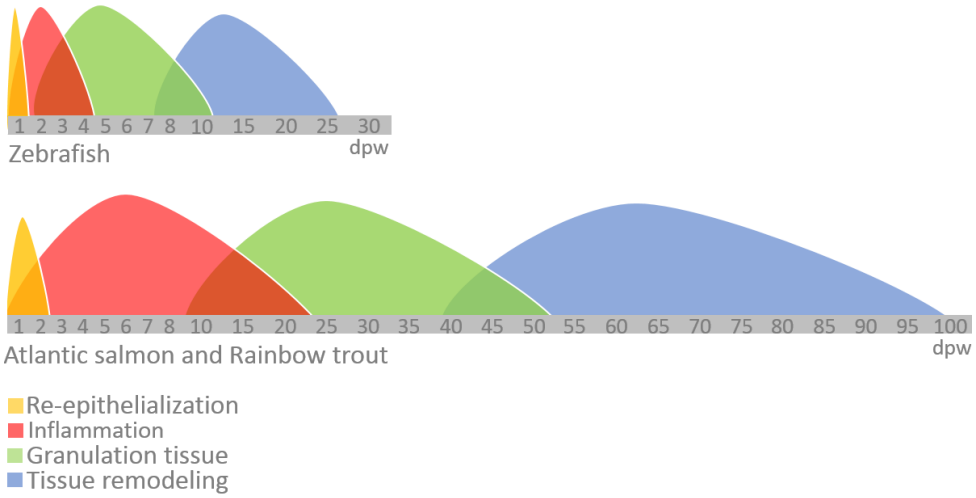


Figure 6. Healing of deep cutaneous wounds in salmonids and zebrafish. Wound healing in fish consists of the overlapping phases, re-epithelialization, inflammation, granulation tissue formation and tissue remodeling. The duration of the different phases vary with life stage, temperature and fish species. The figure is based on publications in table 1, and the results presented in paper III and IV.

In fish, most studies focus on either superficial wound healing or healing of deep cutaneous wounds. The advantage with full thickness wounds (incisional wounds and punch biopsy wounds) compared to other wound healing models (scale loss and abrasion) is that regeneration of both epidermal and dermal structures can be studied. Articles concerning deep cutaneous wound healing in fish, which are currently known to the author, are listed in Table 1. This overview shows that there are great variations in the duration of the wound healing experiments, experimental design and the type of method used to puncture the skin. There are also great variations in how the samples were analyzed, thus it is difficult to directly compare results across articles.

Table 1: Previous studies on full thickness wounds in fish. Fish species, wound type, topic of the study, reference (ref) and duration of the experiment in days post wounding (dpw). Abbreviations: Punch biopsy wound (pb).

Fish species and topic of the study	wound type	dpw	ref
African catfish (*Clarias gariepinus*) Descriptive study of healing processes	10x20mm	30	[84]
Atlantic Salmon (*Salmo salar*) Effect of temperature and zinc on wound healing	5 mm pb	14	[21]
Atlantic salmon (*Salmo salar*) Effect of temperature on re-epithelialization	incisional	21	[85]
Atlantic salmon (*Salmo salar*) Effect of hydrocortisone implants on wound healing	incisional	90	[29]
Common carp (*Cyprinus carpio*) Effect of beta glucans on wound healing	6 mm pb	14	[86]
Gilthead seabream (*Sparus aurata*) Body site and effect on wound healing rate	4 mm pb	15	[87]
Gilthead seabream (*Sparus aurata*) Body site and effect on wound healing rate	8 mm pb	7	[88]
Indian Major Carp (*Labeo rohita*) Role of chromophores during wound healing	incisional	20	[89]
Mrigal Carp (*Cirrhinus mrigala*) Asiaticoside and the effect on wound healing	2 mm pb	30	[90]
Rainbow trout (*Oncorhynchus mykiss*) Effect of beta glucans on wound healing	6 mm pb	100	[91]
Rainbow trout (Oncorhynchus mykiss) Effect of Vitamin C on wound healing	incisional	21	[92]
Rohu (*Labeo rohita*) Descriptive study of the wound surface	incisional	4	[93]
Walking catfish (*Clarias batrachus*) Descriptive study of healing processes	5x3mm cut	35	[94]
Zebrafish (*Danio rerio*) Effect of silver nanoparticles on wound healing	dermal laser	20	[95]
Zebrafish (*Danio rerio*) Characterization of wound healing processes	dermal laser	28	[83]
Zebrafish (*Danio rerio*), Characterization of re-epithelialization processes	dermal laser and scale loss	3	[96]

The different phases of wound healing

In the case of deep cutaneous wounds, the entire wound healing cascade is activated. The re-epithelialization process is triggered immediately by wounding. Keratocytes migrate into the wounded area from the surrounding skin, and as a result the normal epidermis become thinner [22, 96, 97]. The migration stops only when the migrating cell fronts meet each other and therefore the closing incision always happens in the middle of the wound [75, 98].

The role of the inflammatory response is to protect the wound from pathogens, clear the wound from cellular debris and recruit cell types involved in granulation tissue formation and tissue regeneration [83]. The initial immune response is dominated by the innate arm of immunity and involves recruitment of neutrophils and macrophages to the wound site [83]. The duration of the inflammatory response, vary with fish species and temperature (Fig. 6). Transition from the inflammatory to the proliferative phase is identified as a key step to successful healing. In mammals excessive and prolonged inflammation results in delayed healing and increased scar formation [99].

As the inflammation subsides, cell proliferation and formation of granulation tissue takes place. Granulation tissue consists of new capillaries, fibroblasts, myofibroblasts, macrophages, neutrophils, cellular debris and new deposits of extracellular matrix [100]. The time it takes for granulation tissue to develop depends a lot on the fish species and temperature. In Zebrafish granulation tissue is present already at 2 days post wounding (dpw) [83]. In contrast, granulation tissue formation started somewhere between 14 and 42 dpw in Atlantic salmon juveniles reared at 10^{o}C [29]. In Rainbow (*Oncorhynchus mykiss*) trout reared at 15^{o}C fibrous repair tissue was present at the wound margins at 10 dpw [92], while Rainbow trout reared at 8^{o}C showed increased transcription of collagens at 14 dpw [91]. Together, these publications suggest that fibrous repair and granulation tissue formation starts between 7-14 dpw in salmonids.

After granulation tissue formation, the tissue enters the remodeling phase. Due to the short duration of most wound healing experiments in fish (Table 1), the remodeling phase is less documented. One study in Rainbow trout have suggested that the epidermis is able to fully regenerated, while the dermis and underlying muscle tissue was partially regenerated one year after wound infliction [91].

Factors that may alter wound healing

Small skin wounds and fin erosion are common for many farmed fish species and may occur during handling and suboptimal environmental conditions. Therefore, there has been a few studies focusing on factors that may enhance wound healing in fish. Diets

supplemented with zinc led to a more mature epithelial structure in Atlantic salmon post-smolts [21] while vitamin C had a positive effects on the formation of fibrous structures in Rainbow trout [92]. The therapeutic asiaticoside increased wound healing in carp (*Cirrhinus mrigala*) [90] and antibacterial agents such as silver nanoparticles may enhance wound healing in zebrafish [95]. The immunostimulant beta glucan promoted wound healing in Common carp (*Cyprinus carpio*) [86], but the same treatment did not affect wound healing in Rainbow trout [91], indicating that there are differences between fish species and the response to a given treatment.

Several factors may also slow down wound healing in fish. In Atlantic salmon juveniles regeneration of fibrous tissue was delayed in fish with hydrocortisone implants [29], while local immune responses in the skin of gilthead seabream was dampened in fish exposed to scale loss and crowding stress [101]. In mammals, it is well documented that many types of stressors may slow down the wound contraction rate [102-104]. How stress delays wound contraction is unknown, but cortisol is believed to be one of the main factors contributing to retarded wound healing [102]. In murine studies, blocking glucocorticoid function with a glucocorticoid receptor antagonist [104], or by adrenalectomy [105], eliminate the stress-induced delay of wound healing. In mammals, many other factors may also delay wound healing such as age, obesity, malnutrition, diseases and more [106].

2. SCIENTIFIC AIM

The overall goal of this thesis was to improve the understanding on how the skin of post-smolt Atlantic salmon changes in response to external stressors relevant, for post smolt production in S-CCS.

Four main objectives were set:

1. Assess the potential of skin as an indicator of animal welfare during intensified rearing conditions; high biomass and reduced specific water flow (paper I, paper II, paper IV).

2. Expand our knowledge regarding mucin genes in Atlantic salmon and their transcriptional regulation in response to aquaculture relevant stressors (handling stress, high fish density, reduced specific water flow and wounding) (paper I, paper II and paper IV).

3. Develop a standardized wound healing model for Atlantic salmon and describe the cutaneous wound healing process in post-smolt Atlantic salmon (paper III).

4. Identify events in the healing process that may be used as check points to evaluate progression of wound healing and the stress levels in practical salmon farming (paper IV).

3. METHODOLOGICAL CONSIDERATIONS

3.1 Experimental conditions

The work in this thesis is based on the following four experiments:

- Experiment 1: combined fish density and specific water flow study (paper I)
- Experiment 2: combined fish density and acute challenge test experiment (paper II)
- Experiment 3: combined fish density and wound healing study (paper III and IV)
- Experiment 4: acute short-term handling stress experiment (paper II)

All the experiments were performed on post-smolts reared in flow-through systems with seawater. Experiments 1-3 were run at the Industrial and Aquatic Laboratory (ILAB) located at the University of Bergen under similar conditions, while experiment 4 was performed at Nofima Centre for Recirculation in Aquaculture (NCRA) located at Sunndalsøra (Norway).

Experiment 1: combined fish density and specific water flow study

Experiment 1 was designed in order to examine how both stocking density and specific water flow affected animal welfare (paper I). The fish were kept undisturbed except for the daily maintenance routine in order not to cause excess disturbance to the animals. Since the proximity of tanks to e.g. common-use walkways may affect growth indices and stress levels [107], the control groups was held furthest from the entrance. Similar considerations were also taken when designing the other experiments. For further details with regard to experimental design, see paper I and Calabrese et al. [5, 108].

Experiment 2: combined fish density and acute challenge test experiment

Experiment 2 was designed in order to examine the effect of high fish densities followed by an acute challenge test. The experiment was run in the same laboratory and under the same circumstances as experiment 1. Further details concerning experimental design are presented in paper II.

Experiment 3: combined fish density and wound healing study (paper III and IV)

Experiment 3 was designed to identify events in the wound healing process that were affected by a chronic stress. The experiment was run in the same laboratory and under the same circumstances as experiment 1 and 2. As handling procedures are known to be stressful for the fish [14], biomass was not adjusted during experiment 3. Over the time course of the study the fish density increased as a result of growth in the high-density group, while it decreased as a result of sampling in the low density group. As a consequence, both groups may have established different behavioral patterns during the experiment. Fish in the high density treatment were standing in the water column, while fish in the low density treatment had a tendency to be located close to the tank bottom. Towards the end of experiment 3, two incidences of severe tail biting were observed on small individuals (98 and 64 grams) in the low density group. In the high-density group, there were no observed incidences of severe tail biting. Previous studies have reported that fish reared at too low densities may exhibit territorial and aggressive behavior [109, 110]. Thus, the observed tail biting in the low density group likely results from aggressive territorial behavior triggered by low fish density. Aggressive behavior can occur due to underfeeding (Sigurd Handeland pers. comment). However, the fish were fed in excess, both in the high and low-density group.

Prior to experiment 3 great care was taken evaluating different wound healing approaches. The technique should be easy to operate, repeatable and limit harm and discomfort should come to the animal. The whole procedure should also be performed relatively quickly in order to get the animal back in the water. Previously, several wounding techniques have been used in fish, such as incisional wounds (scalpel/razorblade cut), dermal laser and punch biopsy tools of various sizes, as summarized in Table 1. All these techniques damage all the layers of the skin including the muscle. Punch biopsy tools and razorblade/scalpels are easily accessible. The drawback with incisional and punch biopsy wounds is that great care must be given when the wound is inflicted in order to create the same wound depth [75]. The advantage with a punch biopsy wound compared to an incisional wound is that wound contraction can be monitored by different macroscopic imaging techniques [75]. In the

thesis of Jacob Schmidt (Schmidt et al., 2013 and personal comment from the author), no changes in behavior or mortalities were observed after wound infliction in rainbow trout with a 6mm punch biopsy tool. In experiment 3, a biopsy tool with a diameter of 5 mm was used, which gives a slightly smaller wound area and possibly causes less discomfort to the fish compared to a 6 mm punch biopsy tool. Prior to experiment 3, it was considered to use smaller punch biopsy tools (2-4 mm). However, due to the size and resistance of the scales, it was easier to penetrate the fish skin with a 5 mm punch biopsy tool compared to 2-4 mm tools.

Another relevant question to ask when conducting potentially painful experimental procedures is whether the animal experiences pain and distress. According to the legislation for the protection of animals used for scientific purpose, the procedure performed on the animal has to be classified as non-recovery, mild, moderate or severe [111]. Procedures done on animals where the animals are likely to experience short-term mild pain, suffering or distress are classified as mild. Procedures, which are likely to cause moderate impairment of the wellbeing or general condition of the animals are considered as moderate. While procedures, which are likely to cause severe impairment of the wellbeing or general condition of the animals are considered severe. Superficial biopsies such as ear and tail biopsies in mammals are examples on mild procedures [112]. For this reason a variety of wound healing models, including punch biopsy techniques are also used in human studies [113]. Thus, there are reasons to claim that skin biopsies in fish also should be regarded as a mild to moderate procedure. Nevertheless, it is not unlikely that the fish will experience pain and distress both during the procedure and also after the procedure. Therefore, we strived to minimize the number of fish with skin biopsies. For this reason, the experimental unit was kept to one tank. To reduce potential tank effect, the tanks were located next to each other in the rearing facility with water and oxygenation regulated via the same systems.

Experiment 4: acute short-term handling stress experiment

Experiment 4 was designed in order to detect any effects of handling stress (netting and air exposure) on mucin transcription in the gills, skin and intestine. Post-smolts were reared under standard conditions (25 kg/m^3) in a flow-through system with 32 ppt. seawater at NCRA. Fish were individually netted and exposed to air for 30s followed by 60s recovery in oxygenated water. The procedure was repeated three times. The samples were collected 3h and 24h post stress. See paper II for further details.

3.2 Histology, micro- and macro anatomy

In this thesis, established histological techniques such as light microscopy and scanning electron microscopy (SEM) were used to examine the microanatomy of the skin. In addition, in paper I whole mount fluorescent histochemistry was used and further developed for visualization of the epithelial surface on skin samples. In paper III and IV, macro photography of unstained skin samples was used to follow the progression of wound healing.

The whole mount fluorescent staining technique (Paper I) relies on direct staining of the tissue samples with fluorescent labelled dyes. In fluorescent histochemistry, specific tissue structures can be targeted and visualized with specific dyes and/or antibodies. A much higher magnification and resolution may be obtained with SEM. Choice of technique depends on the goal of the analysis and the level of details that are required. However, since both methods visualize the surface of the skin, other techniques must be used to assess deeper tissue structures.

Macro photographs (Paper III and IV) of unstained tissue samples were taken. In the late wound healing phase, dense connective tissue appeared as thick grey bands, easily separated from the rest of the granulation tissue on the macro photographs. Melanocytes were also clearly visualized on the macro photographs, both on cross sections and on horizontally oriented samples. Since the melanocytes have a large size,

much of the cell and the overall architecture was not visual on thin tissue sections. Thus, it was easier to locate the melanocytes on the macro photographs compared to tissue sections stained with e.g. Fontana Masson, a melanin specific dye. Based on these findings, the macro photographs can be used to evaluate formation of dense connective tissue and melanocyte migration during wound healing. Preferably, the technique should be used together with other staining techniques to verify the findings.

3.3 Gene transcription analysis

Transcription is the first step of gene expression where a gene is copied into mRNA. The mRNA transcripts can be measured with a variety of methods such as RT-qPCR, microarray, nucleic acid sequence-based amplification (NASBA) and RNA-sequencing. In this thesis, RT-qPCR and microarray were used to measure transcriptional responses. Both methods have certain advantages and limitations.

Microarray

In experiment 3, a 15k oligonucleotide microarray [114], was used to study gene expression in response to wounding and chronic stress (paper III and IV). In genome wide analysis (such as microarray), where thousands of genes are measured, the risk of detecting false positives are high [115]. To detect "true" changes, and reduce the risk of false positives, a sampling time-line may be used, such as in experiment 3 where seven sampling points were included in the analysis. Only genes that were significantly different from the control at three our more time points were included in the results describing the cutaneous wound healing process (paper III). Thus, the identified differentially expressed genes (DEG) are likely activated by wounding, and not by other factors that may temporarily affect gene transcription. The drawback with this approach is that DEG relevant for only one or two time points may be lost.

Functional annotations of genes are of great importance for the interpretation of microarray results. Both Gene Ontology (GO, Gene ontology consortium, 2018) annotations and Nofima's own annotations, Salmon and Trout Annotated Reference

Sequences (STARS) database [114], were used to interpret the data presented in paper III and IV. At present, GOs is the most widely used vocabulary of gene functions. The drawback of GOs is that annotations for many taxa (including Atlantic salmon) are based on automatic methods which may cause "incorrect" GO predictions and vocabulary [116]. Currently, the most widely used tool to annotate non-model organism is Blast2GO, which relies entirely on sequence similarities to "known" species [117]. This approach is useful, but error prone. First, the functions of many genes might change in the course of evolution and therefore sequence similarity does not always imply orthology [116]. Furthermore, annotations are incomplete in the way that they often lag behind accumulation of knowledge [116].

A similar procedure to the GO annotations are implemented in Nofima's bioinformatic pipeline STARS [114]. The vocabulary in STARS was composed to cover the key topics in aquaculture research with a small number of terms. In STARS the genes are annotated mostly manually using information from public databases (GO, KEGG and Uniprot), scientific publications and results from own experiments. Currently (April, 2018), STARS contain results > 3400 microarray analyses representing a very important source of knowledge. Functional annotations are retrieved together with gene expression data, which greatly assists in mining of the results. STARS are used internally and this means a certain limitation for publications.

STARS annotations were used for gene set enrichment analysis in paper III and IV. Gene set enrichment analysis is a method to identify gene classes that are over represented in a given data set. The aim of the enrichment analysis is to retrieve a functional profile of the gene set in order to better understand the underlying biological processes. This can be done by comparing the input gene set to each of terms in the GO database or in our case the STARS database. As explained above, STARS annotations are based on a solid database form salmon and trout research, while the GOs are based on existing literature from many species. Therefore, we chose to present the data in paper III and IV with STARS categories. To avoid any misconceptions a

full list with gene ID, gene name and their respective STARS category was submitted together with the manuscripts.

RT-qPCR

In experiment 2 and 4, the goal was to investigate the transcriptional response of the identified mucin genes in response to handling stress and acute challenge test. The aim of the study was to identify changes in the transcriptional level of a relative small number of transcripts (the mucin genes) on a relative large number of samples, thus RT-qPCR was a suitable method. In experiment 1, RT-qPCR was used in order to detect molecular changes in the skin in response to high fish densities. The genes targeted by RT-qPCR had previously been highlighted as "responsive" genes in the skin of Atlantic salmon [118, 119]. For this experiment, microarray would potentially have provided a deeper understanding of the ongoing transcriptional responses in the skin. RT-qPCR is cost effective and was therefore chosen as the analytical method.

Primer design is a very important step when running a RT-qPCR reaction. Prior to the release of the Atlantic salmon genome [120], thus prior to the work with identifying the mucin genes, primers were designed for the mucin-like genes *mucin-like 2* and *mucin-like 5* (paper I). These primers were based on the salmon genome available at that time [121]. After acquiring the identification of the seven mucin genes in the new Atlantic salmon genome [120] (paper II), it was possible to investigate whether the primer pairs for the *mucin-like 2* and *mucin-like 5* genes were binding any of the identified mucin genes. These results showed that the primer-pair for *mucin-like 5ac*, bind the mucin sequence presented as *mucin5ac.1* with accession XP_013982550.1 in paper II. Thus, it is quite certain that the *mucin-like 5ac* primer-pair used in paper I binds an actual mucin. In contrast, a blast search with the primer pair used for *mucin-like 2* in paper I, showed that these primers binds six different mucin-like transcripts (XM_014188090.1, XM_014182768.1, XM_014188348.1, XM_014182333.1, XM_014188112.1, XM_014127092.1), none of them identified as mucin gene in paper II. Thus, it is highly uncertain that this primer pair binds an actual mucin.

For paper II, primers were designed towards six out of the seven identified mucin genes. Due to high sequence similarities between the *mucin2.1* and *mucin2.2,* and *mucin5ac.2* and *mucin5ac.4*, it was not possible to design primers which separated between these genes. The *mucin2.1* and *mucin2.2* shared a 99% sequence similarity, similarly *mucin5ac.2* and *mucin5ac.4* shared a 99% sequence similarity (blastn suite-2sequences). Thus, the primers designed for these sequences bind both of the *mucin2* variants (*mucin2.1 and 2.2*) and both *mucin5ac.2* and *mucin5ac.4*. As a result, five primer pairs binding six different mucin genes were used in this thesis.

4. Results and discussion

4.1 Effects of high production intensities on intact skin

The aim of experiment 1 was to investigate the effect of fish density and specific water flow on skin health in post-smolt Atlantic salmon reared in flow-through systems with full salinity (paper I). Fluorescent histochemistry of the epithelial cell surface was used to evaluate whether high fish densities (25, 50, 75, 100 and 125 kg/m^3) and low specific water flow (0.2, 0.3, 0.4, 0.5 l/kg/min) affected the amount of mucus, number of surface mucous cells or caused damage to the epithelial layer. The fluorescence staining was combined with transcription analyses (RT-qPCR) of genes known to be affected in skin during various stress conditions.

Microscopic examination of fluorescence stained whole-mount skin samples demonstrated differences in epithelial cell morphology with increased spacing between epithelial cells in fish reared at densities 50 kg/m^3 and 125 kg/m^3 (paper I). The gene expression analysis revealed increased transcription of *mucin-like 2*, *cathepsins B, -D, -L, matrix metalloproteinase 9* and *claudin 10* in fish reared at a density of 125 kg/m^3, while only *matrix metalloproteinase 9* and *claudin 10* had increased transcription at a density of 100 kg/m^3. These results were interpreted as activation of immune and wound healing mechanisms in the skin.

Data from experiment 1 was also presented in two other papers which are not part of this thesis [5, 108]. This data showed that the specific growth rate was significantly reduced at a stocking density $\geq$ 50 kg/m^3, as described by Calabrese and colleagues [108]. When the fish density increased from 100 kg/m^3 to 125 kg/m^3, a 42 % decrease in specific growth rate was observed over the eight weeks trial [108]. Further relevant for the findings in paper I, a stocking density of $\geq$ 100 kg/m^3 led to increased pelvic fin damage and higher prevalence of cataracts in the 125 kg/m^3 treatment [108]. As the fins are protruding from the fish, these are more prone to erosions than the body. In fact, fin erosions are the most reported welfare issue in Rainbow trout reared at high

fish densities [122]. Previous studies on Atlantic salmon have found that densities above 22 kg/m^3 (in the range 9.7 to 34 kg/m^3) can be associated with reduced fin conditions [109], and fish reared at densities below 30 kg/m^3 have less pronounced fin damage [110]. Given the findings presented in paper I, fin erosion may indicate increased risk of epithelial cell damage not only on the fins but also on the main body of the fish.

Several studies have investigated the effect of fish density on the growth of Atlantic salmon [108, 123-125], however none of these studies have included molecular or histological evaluation of the skin. In general, results from the fish density studies are difficult to compare because they operate with different density groups, different density ranges and different life stages [126]. Nevertheless, a review by Thorarensen and Farrell (2011) concluded that densities up to 80 kg/m^3 do not limit the growth and survival of Atlantic salmon post-smolts. Further relevant for the findings in paper I, post-smolts are using energy to adapt to the marine environment (reviewed by [18, 127]), thus post-smolts are more vulnerable for diseases [128, 129]. Gradual morphological development in the skin with a delayed recovery of immune functions has also been observed in post-smolts after sea transfer [24]. Therefore, post-smolts may be more sensitive to external factors such as high fish densities compared to other life stages.

Reduced specific water flow also activated stress responses in the skin of Atlantic salmon (paper I). After eight weeks, transcription of *mucin-like 2* and *-5ac, inducible nitric oxide synthase, lysozyme* and *cathepsin B* and *-L* increased in skin samples from fish reared in tanks with a specific water flow of ≤ 0.3 l/kg/min. Increased transcription of these genes implied activation of stress and immune responses in the skin at low specific water flow. Both total ammonia nitrogen (TAN) and CO_2 levels increased as conditions intensified. The mean CO_2 concentration in the water was three times higher in the lowest (~15 mg/l) compared to the highest flow rate (~5 mg/1) [5]. This was reflected in increased levels of pCO_2, and HCO_3, and decreased pH in the blood of fish reared in the lowest specific water flow treatment [5]. These are normal physiological

responses which are activated by increased CO_2 levels [130]. Fish reared at the lowest specific water flow levels also had higher oxygen consumption and the authors suggest that the physiological responses triggered by reduced specific water flow are energy costly [5]. However, there were no effects of low specific water flow on the measured external welfare parameters (skin, fins and eye). As a conclusion, the observed activation of stress and immune responses in the skin are likely a result of the reduced water quality parameters at low specific water flow.

In experiment 3, control samples were taken from intact tissue from fish reared at both high and low fish densities. At time point 0, the sampling before wounding, the fish density was 116 kg/m^3 in the high fish density tank and 22 kg/m^3 in the low fish density tank (paper IV). Also in this experiment, high fish densities activated immune responses in the skin of Atlantic salmon post-smolts. In total 34 genes were up-regulated by high fish densities (log_2 expression ratio > 0.8, $p < 0.05$), among these genes 18 was annotated with immune functions (Fig 7 a and Table 2). Hence, the high fish density treatment in experiment 1 and in experiment 3 activated immune responses in intact skin of Atlantic salmon. In paper IV, it was further demonstrated that the overall inflammatory response to wounding was enhanced by chronic stress (paper IV). The effect was strongest at 3 dpw, with 107 DEG involved in diverse immune functions being enhanced by stress (Table 2). These results imply that early signs of activated immune responses in intact skin may have major consequences on the overall immune response if the fish is wounded.

Table 2: Chronic stress escalates the inflammatory response. The number of differentially expressed genes (DEG) being up-regulated at high fish densities and classified as "immune" in the STARS database. Data collected from the microarray analysis conducted in experiment 3. Time point 0 represents intact skin. Genes with a log_2 expression ratio > 0.8 (1.75 fold change) and $p < 0.05$, were considered significantly different from each other

Days post wounding	**0**	**1**	**3**	**7**	**14**	**36**	**43**	***57***
DEG with "immune" functions	18	8	107	41	40	24	39	35

a ***Effect of high fish density on intact skin***

Gene name	*Symbol*	dpw
Immunity		0
Serum amyloid A	*saa*	
serum amyloid A	*saa*	
Serum amyloid A5	*saa*	
Serum amyloid A-5 protein	*saa*	
Natterin-like protein	*null*	
MHC class I	*mhc1uxa2*	
C-C motif chemokine 19-2		
C-C motif chemokine 21 precursor		
Receptor transporting protein 3		
Gig2-3	*gig2l*	
Ubiquitin protein ligase E3A, hect domain	*HERC6*	
Radical S-adenosyl methionine	*rsad2*	
Radical S-adenosyl methionine	*rsad2*	
Collagenase 3 precursor	*mmp13a*	
Matrix metalloproteinase-9	*mmp9*	
CD97 antigen precursor	*null*	
NILT4 leukocyte receptor	*null*	
coiled-coil transcriptional coactivator b	*calcoco1*	

Gene name	*Symbol*	dpw
Metabolism		0
ATPase_Ca++ transporting	atp2a2a	
Cytochrome P450 24A1,	cyp24a1	
Matrix metalloproteinase-9	mmp9	
Translation initiation factor IF-2,	mtif2	
FK506-binding protein 5	fkbp5	
Pyruvate dehydrogenase kinase isozyme 2,	pdk2	
6-phosphofructo-2-kinase	pfkfb3	
Differentiation		
Kazrin-A	null	
Phosphatidylinositol glycan anchor	PIGU	
CCAAT/enhancer-binding protein delta-2	CEBPD	
Homeobox protein EMX2	emx2	
Cell		
BCL2/adenovirus E1B interacting protein	null	
DNA replication complex GINS protein PSF3	PSF3	
Telethonin		

b ***Effect of wounding on selected markers***

Gene name		dpw						
Genetic markers paper I	**Symbol**	1	3	7	14	36	43	57
Cathepsin B precursor	*ctsba*							
Cathepsin B precursor	*catb*							
Cathepsin D precursor	*ctsd*							
Cathepsin L1 precursor	*ctsll*							
inducible nitric oxide synthase	*nos2b*							
Lysozyme C II precursor	*null*							
Lysozyme g	*lygl1*							
Matrix metalloproteinase-9	*mmp9*							

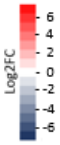


Figure 7. Transcription of selected genetic markers and their response to wounding. a. Differentially expressed genes between intact tissue from fish reared at low and high fish densities. All the genes on the microarray having a positive $\log_2$ expression ratio > 0.8 (1.75 fold change) and $p < 0.05$ is displayed. The results are extrapolated from the microarray data run in experiment 3. **b.** Transcription of similar genes as targeted by RT-qPCR in paper I in response to wounding. Red colour represents up-regulation and blue colour down regulation compared to intact tissue. Results are collected from the microarray data and show $\log_2$ expression ratio in the wounds from fish reared at low fish density relative to intact skin.

The transcriptional responses that were triggered by low specific water flow and high fish densities in experiment 1 implied activation of immune and wound healing responses in the skin (paper I). In experiment 3 the transcriptional response to wounding was measured with microarray (paper III). Therefore the response of the genetic markers used in experiment 1 could be investigated in response to actual wounding (Fig. 7 b). The genetic markers *inos, lysozyme* and *cath b, -d, -l,* were all up-

regulated in the early wound healing phase, while *matrix metalloproteinase 9* were up-regulated in the early and the late phases of wound healing. These finding suggests that both reduced specific water flow and high fish densities trigger transcriptional responses similar to those activated by wounding.

The results presented in this chapter show that early signs of skin damage are difficult to detect through common welfare measurements. However, molecular analyses of skin samples revealed that negative effects are induced and detectable at early stages. Fish farmed at high production intensities show a transcription profile that resembles pathways that are activated by wounding. Further, stress enhances the inflammatory response when the animal is wounded in combination with high fish densities. These findings imply that Atlantic salmon reared under stressful or sub-optimal conditions are subjected to an altered immune response compared to salmon farmed under better conditions, potentially affecting the robustness of the animal. These findings should be considered when operating an aquaculture facility, handling fish and also when examining field samples.

4.2 Mucin transcription and response to the rearing environment

Identification of seven-gel forming mucins

In paper I it was demonstrated that the mucin-like genes responded to both high fish densities and also reduced specific water flow. As a follow-up study, paper II was initiated, with the goal of identifying all gel forming mucin genes in the "new" Atlantic salmon genome [120]. With better genome information available, it was possible to identify seven potential mucin genes (Paper II). The pipeline used for identification of the mucin genes included annotation, domain structure, transcription and phylogenetic analysis. Based on this work seven secreted gel-forming mucin genes were identified. Two genes were annotated as *mucin2* and five genes as *mucin5*. The *mucin2* genes were predominantly transcribed in the intestinal region while the different genes in the

mucin5 family were mainly transcribed in either skin, gill or pyloric caeca. These results were in accordance with transcription profile of mucins found in other fish and mammals [50, 71, 131, 132].

In Table 3, the length of the coding sequence in base pairs, and the length of the translated sequence in amino acids is indicated for each of the seven mucins. From this table it is clear that the coding sequence for *mucin5b* is the longest, with 7898 base pairs (bp) or 2530 amino acids (aa) (Table 3). The rest of the sequences have transcripts in the range of 1661-3754 bp. Only *mucin5b* have the classical domain structure of 3x(VWD-C8-TIL)-PTS-(VWD-C8-TIL), which is conserved in mammalian mucin5s and mucin2s [58]. Except for *mucin5b,* the identified mucin sequences stops where the PTS domains in theory should be located (Table 3). One explanation for this could be that the mucin mRNA transcripts were not successfully aligned to the Atlantic salmon genome [120]. Further, the full size transcript of *mucin5b* is still much smaller compared to related genes in zebrafish. The zebrafish *mucin5ac* (ENSDART00000153708.1) gene features a transcript length of 16267 bps, and a translation length of 5282 aa, while *mucin5b* (ENSDART00000153536) features a transcript length of 18751 bps and a translation length of 6126 aa. The few full-length mucin transcripts that are available in zebrafish have been obtained by cloning the mucin sequences into bacterial chromosomes [131].

Table 3: Gel forming mucin genes identified in paper II. Number of base pairs (**bp**) and number of amino acids (**aa**) in the reference sequence. The predicted domain structure is also indicated in the table. Only *mucin5b* (bold text) has the predicted complete domain structure. The question mark is placed where domain structures are likely to be missing. Information retrieved from NCBI/RefSeq genome.

Gene ID	Symbol	bp	aa	Domain structure
XP_013982550.1	*mucin5ac.1*	2524	819	2x(VWD-C8-TIL)- ?
XP_013981532.1	*mucin5ac.2*	3046	1001	2x(VWD-C8-TIL)-VWD- ?
XP_014037804.1	*mucin5ac.4*	1661	553	2x(VWD-C8-TIL)-VWD- ?
XP_014031311.1	*mucin5ac.3*	2870	946	VWD-C8-TIL-VWD- ?
XP_014031349.1	***mucin5b***	**7898**	**2530**	**3x(VWD-C8-TIL)-PTS-(VWD-C8-TIL)**
XP_014025861.1	*mucin2.1*	2227	759	? -VWD-C8-TIL-VWD- ?
XP_014040158.1	*mucin2.2*	3754	1241	2x(VWD-C8-TIL)-VWD-C8- ?

In conclusion, paper II shows that it is possible to detect Atlantic salmon mucins with a computational pipeline. In the future, sequencing techniques such as nanopore technology with ultra long reads could possibly solve some of the current problems by sequencing the full length of the mucin transcripts [133]. If successful, such an approach would in theory capture all available mucin sequences, and at the same time avoid time-consuming techniques such as cloning into bacterial chromosomes.

Transcriptional regulation of the mucin genes

After having identified the mucin genes, the next goal was to investigate their transcriptional regulation in response to aquaculture relevant stressors. The transcriptional response of the identified mucin genes was investigated on material from experiment 2 (high fish density and acute challenge), experiment 3 (wounding and high fish density) and experiment 4 (acute short-term handling stress). In addition, transcription of *mucin-like 5* (*mucin5ac.1*) was investigated on material from experiment 1 (reduced specific water flow and high fish densities). Overall, the results showed that transcription of the mucin genes responded to all of the above stressors, but the mechanisms triggering, or repressing, mucin transcription are still unclear.

The results suggests that high fish densities trigger mucin transcription in the skin, as *mucin5b, mucin2./4* and *mucin5ac.1* transcription increased after intensive rearing conditions and confinement stress (paper II). Reduced specific water flow also resulted in increased *mucin5ac.1* transcription in the skin (paper I). Given the presented results, it is likely that changes in the water quality parameters may have an impact on the observed alterations in mucin transcription (paper I, II and IV). Due to the metabolism of the fish, CO_2 and TAN levels will increase as the water exchange rate is reduced or biomass increased. Increased biomass and reduced specific water flow may also cause accumulation of particles and bacteria in the water. Several authors have previously demonstrated that different water quality parameters can affect the number of mucous cells. In sea bass (*Dicentrarchus labrax*) both high nitrate concentrations and low oxygen concentrations increased the number of mucous cells in the skin [41]. Increased numbers of epidermal skin mucous cells were noted in brown bullhead catfish

(*Ictalarus nebulosus*), following exposure to acid [42, 43], and water with increased bacterial load introduced changes in the skin mucosal response in common carp (*Cyprinus carpio*) [45].

Transcription of *mucin5b,* and *mucin5ac.2/4* was down regulated in the skin both 3 and 24h after acute handling stress (paper II). These results may imply that perceived stress may have an inhibitory effect on mucin transcription in the skin. In mammals cortisol has an inhibitory effect on mucin transcription (Fig. 4) [51]. As cortisol levels are generally known to increase by acute handling stress [20], similar mechanisms may be present in fish. Further, *mucin5ac.1* transcription was down-regulated in the wounds of chronically stressed fish (paper IV). Histological analysis also showed that the mucus response was dampened in the wounds from fish reared at high fish densities at 7 dpw (Fig. 8a). Several previous studies have suggested that the early mucus response is important to protect the wound during the early healing phase [21, 88, 94]. Thus, if the fish is wounded, high fish densities may reduce the initial mucosal barrier of the fish possibly leaving the wound more susceptible to secondary infections.

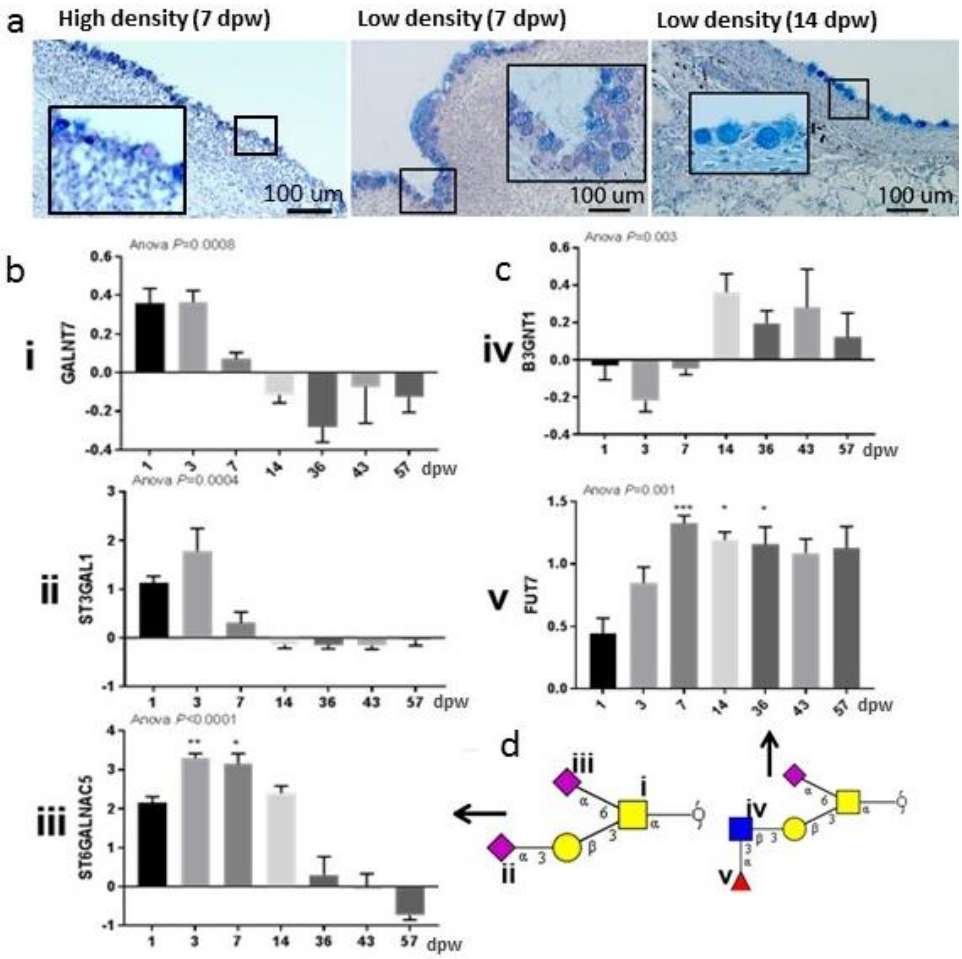


Figure 8: Changes in the mucus composition and glycan biosynthetic pathway. a. Epidermis of wounded salmon reared at high and low fish density at 7 days post wounding (dpw), and at low fish density 14 dpw. Square indicate enlarged area, note the presence of purple mucous cells at 7 dpw. 5µm tissue sections stained with Alcian blue and PAS. **b.** Transcription of *galnt7, st3gal1*, and *st6galnac5* **c.** Transcription of *b3gnt1*, and *fut7*. Log_2FC of glycosyl transferases found on the microarray run in experiment 3. Intact skin was used as control. One-way ANOVA and Kruskal-Wallis H-test, (p-value < 0.05*, 0.01**, 0.001***). **d.** Structural representation of two mucin *O*-glycans in Atlantic salmon skin [53, 54]. Monosaccharides (i–v), and their respective glycosyl transferase is given in panel b and c. Yellow square- *N*-acetyl galactosamine, yellow circle- galactose, blue square- *N*-acetyl glucosamine, red triangle- fucose and purple diamond- *N*-acetyl neuraminic acid (sialic acid). Panel b-d by Dr. Vignesh Venkatakrishnan at the University of Gothenburg.

Correlation of mucin transcription

Looking closer at the transcriptional profile of the different mucin genes it seems like *mucin5ac.1* and *mucin5b* transcription are regulated by similar mechanisms. The genes shared a high positive correlation in experiment 2 (R^2=0.83), in experiment 3 (R^2 > 0.77) and also in experiment 4 (R^2=0.69) (Fig. 9). *Mucin 5ac.1* is located on chromosome 11 and *mucin5b* on chromosome 26 (paper II). However, these data suggest that these genes are regulated by similar mechanisms and both wounding, high fish densities and handling stress may influence their rate of transcription.

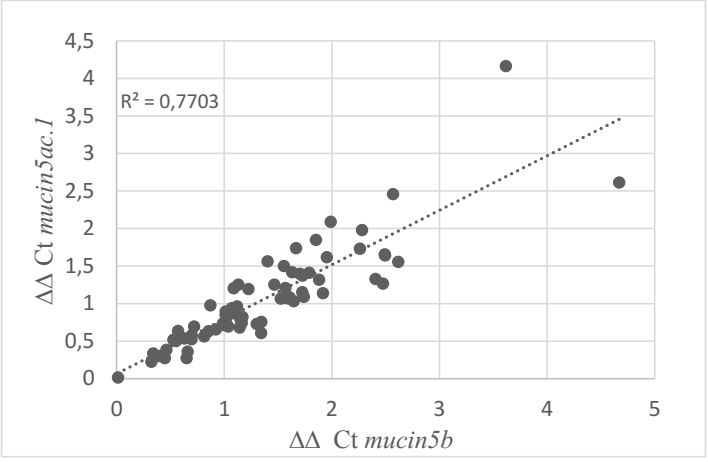


Figure 9: Correlation between *mucin5ac.1* and *mucin5b* transcription. Scatter plot showing the correlation (R^2) between *mucin5ac.1* and *mucin5b* transcription in experiment 3. The samples were normalised to control (low fish density + high fish density) (Paper IV).

Transcription of *mucin5ac.2/4* was not correlated to *mucin5a.1* and *mucin5b* transcription in any of the experiments. Thus, this gene may be regulated in a different way than the two other mucins. An interesting observation regarding *mucin5ac.2/4* transcription was the transcriptional response in experiment 3. Transcription of this gene peaked at 7 dpw (paper IV), at the same time the acute inflammatory response in the wounds also reached its maximum (Paper III). Thus, inflammation may trigger *mucin5ac.2/4* transcription.

Glycosylation during the early healing phase

Mucin transcription is not the only factor which may impact the properties of the mucus gel. With the help from the research group "Mucins in Infection and Cancer" at the University of Gothenburg, transcription of glycosyl transferases present on the microarray were analysed (Fig. 8 b-d). In general, transcription of the glycosyltransferases followed a similar pattern in wounds from chronically stressed fish and control wounds. The enzyme initiating the mucin glycan synthesis, *galnt7*, was slightly increased during the first 3 dpw in both groups, and two sialyltransferases, *st3gal1* and *st6gaknac5*, were up-regulated the first 14 dpw (Fig. 8b). Concurrently, the expression the transferases, *b3gnt1* and *fut7*, decreased (Fig. 8c). This might indicate that the mucins carry different glycan chains during the early wound healing phase. Further, as presented in paper III, the number of purple mucous cells peaked at 7 dpw, and at the later time points (14-57 dpw) there were very few purple mucous cells present in the epidermis (paper III). A different colour in the mucous cells also indicate a different glycosylation pattern of the mucin backbone [53, 54]. At 7 dpw the mucus gel was also sticking to the epidermal surface (Fig. 8a). Thus, a more adherent mucous gel was suggested. Overall, these results suggests that both mucin transcription and glycosylation pattern are altered in the early healing phase, resulting in a more adherent mucus layer.

4.3 Wound healing and the effect of chronic stress

The aim of experiment 3 was to assess the effect of chronic stress on wound healing capacity in the skin of post-smolt Atlantic salmon (paper IV). Skin biopsies (5 mm) were taken from behind the dorsal fin and the wound healing process was followed over a two month period (paper III and IV). High fish density ($\bar{x}$ = 126 kg/m^3) was used as a chronic stress factor and low fish density ($\bar{x}$ = 14 kg/m^3) as control.

The healing process was dominated by an early acute inflammatory phase (1-14 dpw) and a late healing phase with fibrous repair (36-57 dpw) (paper III). The early acute phase was dominated by haemostasis, acute inflammation and epidermal repair, as shown through imaging, histological evaluation and transcriptomics. Most of the

immune genes showed decreased transcription after two weeks, approaching the levels of intact skin. This was also reflected in the tissue sections where reduced inflammation in the wound bed was observed. The transcriptional events further suggested recruitment of lymphocytes during the acute phase, with activation of humoral responses from 14 dpw and onward. The histological results, suggested a more adherent mucus layer, which correlated with altered transcriptional pattern of the glycosyltransferases. The late healing phase (36-57 dpw) was characterized by wound contraction, and formation of granulation tissue and scarring. The proliferative activity in the wound bed was greatest at 36 dpw. As the proliferative activity in the wound bed decreased (43 dpw and 57 dpw), the collagen fibres were aligned in parallel to the epidermal structure. The transcriptional events showed that several genes involved in collagen synthesis, fibril maturation and growth factors peaked in transcription levels in the late healing phase. This orchestrated wound healing process involving re-epithelialization, inflammation, innate and adaptive immune response, tissue regeneration and tissue remodelling, is comparative to dermal wound healing in zebrafish and mammals [83, 134].

As paper III focused on the general wound healing responses, paper IV highlights the effects of chronic stress on wound healing. All samples were analyzed in the same way as described above, with photography, histology and microarray analysis. Both the transcriptional and histological results showed that chronic stress delayed several wound healing responses. On the histological level, high fish density delayed re-epithelialization, the mucus response, scale mineralization, pigmentation and fibrous repair (Fig. 10). At the transcriptional level, inflammation was enhanced while several genes involved in tissue repair such as cell proliferation, mucus responses, collagens and growth factors were transiently repressed by chronic stress. Chronic stress also altered the overall wound morphology and wound contraction. Wounds from the control group were contracting in a more elongated manner compared to wounds from fish reared under chronic stress conditions.

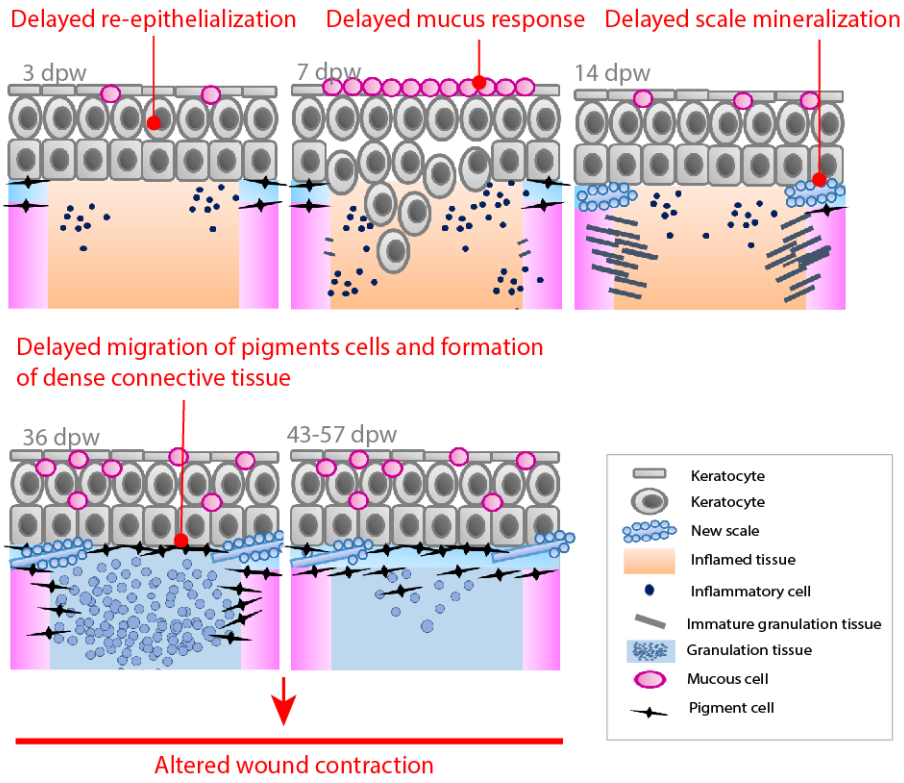


Figure 10. Histological and morphological effects of chronic stress on wound healing. The wound healing process was dominated by inflammation (3-14 dpw) and tissue repair (36-57 dpw). Chronic stress resulted in many temporary histological effects, highlighted with red text. The figure is extrapolated from results in paper III and IV.

From human and other mammalian studies, it is well documented that chronic stress situations are associated with delayed wound healing. As an example, healing of a 3.5 mm biopsy wound took 9 days longer in caregivers of Alzheimer patients compared to controls [135]. Several other studies report similar results and high cortisol levels is believed to be the main cause of delayed wound contraction [104, 105, 136-138]. To our knowledge, only one study has been conducted that investigates the effect of chronic stress on wound healing in fish [101]. This study focused on epidermal repair and scale regeneration in gilthead seabream. The presented results showed suppression of seven genetic markers involved in wound healing. However, the authors did not find any histological differences between stressed and unstressed fish. Further, it can be argued that low water temperatures may cause stress to the fish. The optimum rearing

temperature for post-smolts is 12-14ºC, and both higher and lower temperature may result in reduced growth rate [139]. In this context, it is relevant to mention that low temperatures resulted in delayed epithelial repair in Atlantic salmon [21], cloud minnow (*Tanichthyes aulbonubes*) [85] and plaice (*Pleuronectes platessa*) [22]. In paper IV it was demonstrated that low temperatures resulted in longer pseudopods on primary keratocytes in cell culture, and the keratocytes in the epidermis of chronically stressed fish showed similar features (paper IV). Thus, this phenotype may be a response to reduced mitotic activity, a response to environmental stress or both.

Wounding changes dietary requirements

The results from experiment 3 presented in paper III and IV mainly focused on the effect of inflammation and tissue regeneration on wound healing. However, this was not the only effect of wounding. The microarray data also showed effects on other pathways, including genes involved in metabolism, which in general was down regulated by wounding (Fig. 11).

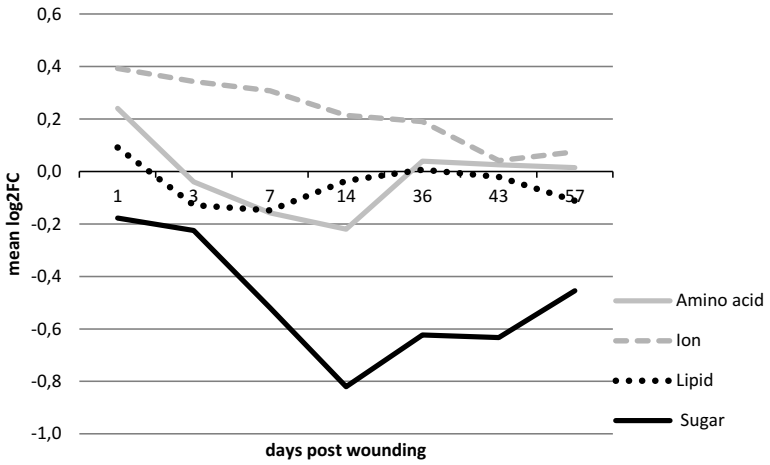


Figure 11. Metabolism in healing wounds. Each line represents the mean transcription level (in the control group) of all differentially expressed genes (DEG) within the following functional STARS category: amino acid, ion, lipid and sugar metabolism. The results are extrapolated from the microarray data generated in experiment 3.

Within each metabolic group, there were several genes enhanced by wounding, including zink transporters and metabolism of the amino acids arginine, glutamate and glutamine (Fig. 12). In mammals, it is well documented that both zink and arginine/glutamine may influence wound healing [140-143]. Little literature exists on these nutrients and wound healing in fish. One study in Atlantic salmon have reported positive effects of zinc on wound healing and epidermal repair [21]. Wounding also resulted in up-regulation of several genes involved fatty acid metabolism (Fig. 12). As an example, *perilipin* an important regulator of lipid storage, and fatty acid elongases which are important for the production of very long chained fatty acids found in cell membranes, were up-regulated by wounding [144]. In mammals, several studies have reported effects of omega-3 fatty acids on wound healing [141, 145, 146], thus similar effects may be present in fish.

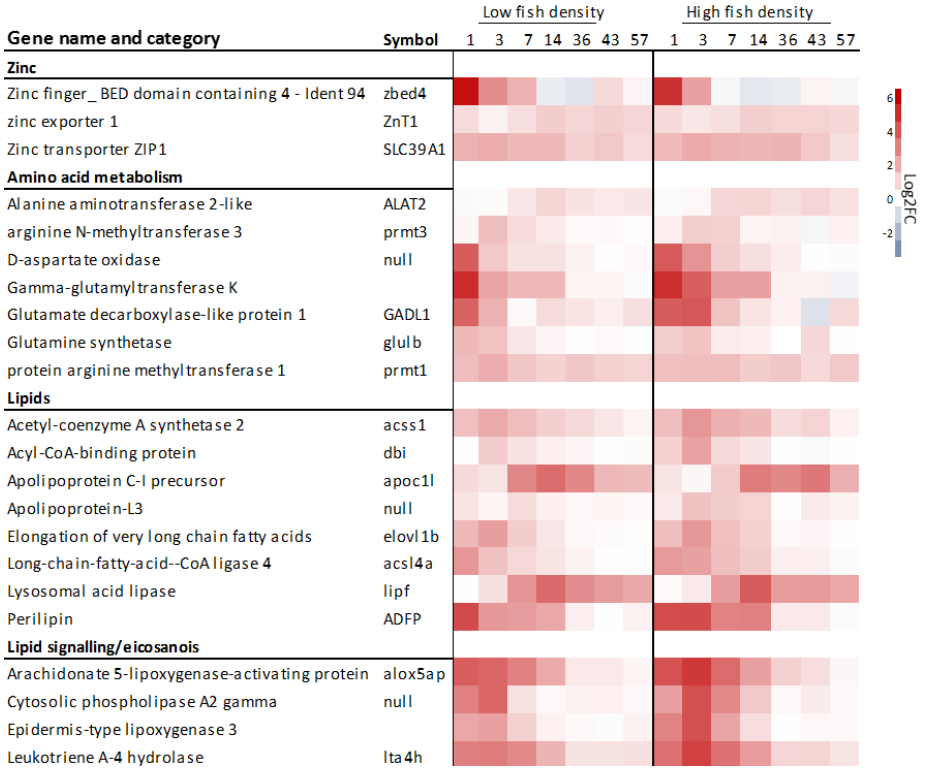


Figure 12. Wounding enhanced transcription of genes related to metabolism and lipid signaling. Selected differentially expressed genes involved in amino acid and lipid metabolism, lipid signaling and zinc transportation**.** Data collected from the microarray results generated in experiment 3.

Many lipid signaling molecules (eicosanoids) were also up-regulated by wounding and further enhanced by stress (Fig. 12 and paper IV), thus it could be hypothesized that high levels of omega-3 fatty acids would reverse some of the adverse effects that chronic stress had on wound healing. A previous study in Atlantic salmon showed that decreased levels of omega-3 fatty acids in the diet resulted in replacement by pro-inflammatory n-6 fatty acids, and this feature was most markedly in the skin [147]. Further, diets containing high levels of omega-3 fatty acids have been used as a tool to control viral inflammatory diseases in Atlantic salmon by modulating tissue fatty acid composition, eicosanoid production and immune functions [148-150].

The wound healing model and analytical tool box developed in this thesis have already been used in a project on dietary requirements for skin, gut and gill health, financed by the Research Council of Norway (project number: 901265). One of the work packages in this project investigated the effect of dietary supplements of omega-3 fatty acids and zinc on cutaneous healing. Preliminary results from this trial suggest that both omega-3 fatty acids and zinc changes the histological appearance of the wound and also the wound contraction rate [151]. Wounds in fish fed high levels of zinc healed faster compared to fish fed with a low zinc diet. The groups that were fed low levels of omega-3 fatty acids had the overall slowest wound healing rate.

Meta-analysis reveals similarities between infections and cutaneous healing

Meta analysis is a broad term meaning comparison across large data sets. In order to investigate similarities in the transcriptional responses from different experiments, a meta-analysis is normally carried out after an experiment is incorporated into the STARS database. To assess p-values, STARS run enrichment analysis in the same way as for GO and KEGG. Meta-analysis with data from experiment 3 showed that more than 50 previous experiments had an overlap with > 50 % of the DEG in the respective study also being DEG in experiment 3. Among the studies shearing transcriptional responses similar to those activated by wound healing were viral challenge trials [152, 153], INFα transfected plasmid injection in the muscle [154], black spots in the muscle filet [155], and skin infected with salmon lice [118] (Table 4).

Table 4: Meta-analysis. The table shows an overview of selected experiments that were compared with the wound healing experiment using the meta-analysis tool in the STARS database. The total number of differentially expressed genes (DEG) in the selected experiment is shown in the DEG column. The cut off for the individual experiment is indicated with p-value and fold change (FC). The overlap (OL) column shows the number of overlapping DEG with the wound healing experiment. The immunity (IMU) column shows the percentage of the overlapping DEG that are annotated with immune functions. The results are extrapolated from STARS. Abbreviations: *Salmonid alfavirus* (SAV).

EXPERIMENT	TISSUE	CUT OFF	DEG	OL	%OL	%IMU	REF
Black spots	Muscle	p<0.01, FC> 2	966	749	78	25	[155]
Plasmid	Muscle	p<0.05, FC>1.75	1038	739	71	26	[154]
SAV	Heart	p<0.01, FC>1.6	850	599	70	35	[152]
Skin lice	Skin	p<0.01, FC>1.75	772	457	59	7	[156]
Wound healing	Skin	p<0.05, FC> 1.75	4292				

It is not surprising that the wound healing study overlaped with the transcriptional response activated by salmon lice infected skin. The salmon lice feed on skin and mucus causing both superficial and in some cases deep wounds [157]. Further, the *Salmonid alphavirus* (SAV) is a systemic viral disease characterized by necrosis and loss of exocrine pancreatic tissue, myocarditis and skeletal muscle degeneration. As demonstrated by Johansen et. al. 2015, SAV activated early innate immune responses, such as the interferon (INF) axis in parallel with diverse humoral and cellular responses [152]. IFNα containing plasmid injected to the muscle also mediated an increase in gene transcripts of the IFN axis, chemokines and markers for leukocytes [154]. Similar responses were also activated by wounding (paper III). Overall, these results suggests that the inflammatory responses, being of viral or parasitic origin, activates similar inflammatory mechanisms as mechanically wounded skin. This makes the wound healing model a promising tool to separate between inflammatory responses triggered by wounding to those triggered by infection.

The study with the overall greatest overlap with the wound healing experiment were dark spots in Atlantic salmon muscle (Table 4) [155]. A total of 966 DEG were found in Atlantic salmon filet with black spots compared to control filet [155]. Out of these DEG, 78% were overlapping with wound healing experiment. Black spots are

characterized by accumulation of melanomacrophages in the filet, resulting in down-grading of the final product. Previous literature has suggested a combination of inflammation induced with trauma, vaccination [155], or infection with *Piscine orthoreovirus* [158] to be involved in the development of black spots. Initially, development of black spots occur as red focal areas with intramuscular bleeding, which over time may develop into a melanized phenotype [159, 160]. Similarly, wound healing starts with bleeding and inflammation, which is later replaced by fibrosis and melanocyte infiltration (Fig 10 and Fig. 13). Based on the findings in paper III, the melanocytes are infiltrating the wounded area between 14 and 36 dpw. This is not a new finding, and hyperpigmentation is demonstrated in healing wounds from many fish species [83, 86, 91, 101]. Thus, hyperpigmentation is a natural part of the healing process. Therefore, increased accumulation of melanomacrophages in the filet, may be a natural part of the ongoing repair process in response to previous trauma.

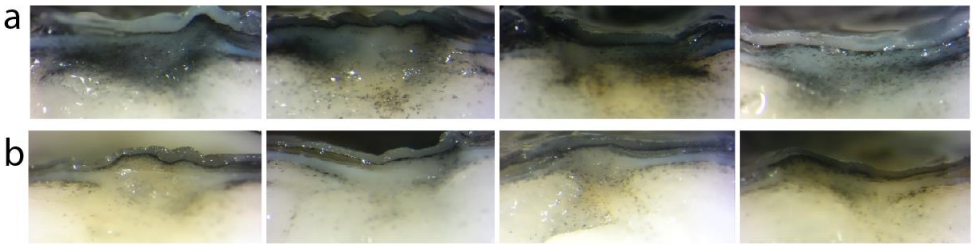


Figure 13. Stronger pigmentation in wounds from chronically stressed fish. a. Wounds from four different individuals reared under chronic stress conditions, 57 days post wounding (dpw) **b.** Wounds from four different individuals, control group 57 dpw. Samples were collected in experiment 3. Stereoscope pictures, 16X.

What is further interesting is that the chronically stressed fish in experiment 3 had a darker skin color compared to the control fish and the wounds healed with a stronger pigmentation (Fig. 13). This darker coloration could simply be an adaptation to the high density treatment and the light conditions in the tank. Another contributing factor could be overproduction of the α-melanocyte-stimulating (α-MSH) hormone brought on by the stress response [161-164]. Cortisol may also change skin pigmentation, as the dark color of senegalese sole (*Solea senegalensis*) was explained by elevated cortisol concentrations [165]. In humans, it has also been demonstrated that the stress

hormone adrenocorticotropic hormone (ACTH) results in hyperpigmentation of the skin [166]. Thus, many hormones involved in the stress response may enhance skin pigmentation. Whether chronic stress results in enhanced pigmentation in black spots in Atlantic salmon filet needs further investigations.

In conclusion, the transcriptomic response to parasitic and viral infections in skin, muscle and heart are similar to the transcriptional response triggered by cutaneous wound healing. Thus, it is likely that chronic stress will alter the inflammatory response and delay repair processes in infected tissue. It is also likely that the diet will affect the outcome of the healing process. These findings may be implemented during operational farming practices, and farmers must strive to avoid conditions that cause excess stress to the fish.

5. Conclusions

This thesis has demonstrated that different stressors can change the skin, mucus composition and wound healing progression in Atlantic salmon. The different stressors that were employed were high fish densities, acute challenge test, reduced specific water flow and acute handling stress. Based on the findings in this thesis, the following main conclusions can be stated:

- High fish densities may cause damage to the skin and trigger responses resembling wound healing mechanisms. Similarly, reduced specific water flow activates transcription of stress and immune responses in the skin. These changes are not visible upon general welfare examinations of the skin.

- Seven secreted gel forming mucins were successfully identified in the Atlantic salmon genome. Factors that may change mucin transcription are handling stress, inflammation, high fish densities and low specific water flow. Thus, a wide range of factors may regulate mucin transcription.

- Wound healing in post-smolt Atlantic salmon follows the classical vertebrate wound healing cascade, with inflammation and tissue repair. Several checkpoints may be used to evaluate the wound healing process in Atlantic salmon and the influence of stress, these are: the re-epithelialization process, mucus response, scale formation, wound pigmentation, fibrous repair, wound contraction rate and the overall wound morphology.

6. Future perspectives

In order to use mucin transcription and mucus secretion as diagnostic tool, future studies should focus on identification of all the mucin genes and better characterization of the mucous cells. Identification of the mucin genes could be accomplished by new sequencing methods, giving longer and better reads. This being done, similar approaches as in paper II could be used to characterize how these genes are regulated in response to a wide range of stressors. Further, it is not unlikely that mucus production is correlated with resistance to pathogens. Thus, identification of the mucin genes could be of great importance to breeding companies that are looking for the genetic variation explaining resistance. Further, better characterization of the mucous cells themselves, with single cell RNAseq sequencing and in-situ hybridization would aid in better understanding of these cells, what they produce and factors which may alter their composition.

As highlighted in the result and discussion, future studies should focus on strategies that may promote skin robustness and wound healing processes. The effect of dietary components on wound healing and skin robustness has been carried out and future studies are planned. Other factors may also influence wound healing. One example is circadian rhythms, which have major influence of the wound healing speed in mammals. If similar effects are present in fish, it may have consequences for practical farm management, e.g. when rough handling procedures, such as de-lousing, should be carried out. In this context, it would also be interesting to investigate different light regimes and wavelengths on wound healing properties, this could be of particular interest in CCS where the light regime is strictly controlled. Several previous studies have also found that the tank color affects production of stress hormones in fish. Thus, it could be relevant to investigate the effect of tank color on the stress response and the impact on wound healing. In this context tank enrichment, in general and also the presence and impact of cleaner fish could be targets for future studies.

Source of data

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Impact of fish density and specific water flow on skin properties in Atlantic salmon (*Salmo salar* L.) post-smolts

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ABSTRACT

Prolonged production of Atlantic salmon (*Salmo salar*) post-smolts in closed-containment systems has prompted research into biological requirements under higher production intensities. This study examined the effect of fish density and specific water flow on skin health in post post-smolts particularly focusing on epithelial cell morphology and gene expression.

In the density experiment, post-smolts were kept at five different fish densities (25, 50, 75, 100 and 125 kg/m^3) at a specific water flow rate of 0.6 l/kg/min. Microscopic examination of fluorescence stained whole-mount skin samples demonstrated differences in epithelial cell morphology with increased spacing between epithelial cells at 50 kg/m^3 and 125 kg/m^3. Gene expression analysis revealed increased transcription of *mucin-like 2*, *cathepsins B, -D, -L*, *matrix metalloproteinase 9* and *claudin 10* in fish reared at a density of 125 kg/m^3, while only *matrix metalloproteinase 9* and *claudin 10* had increased transcription at a density of 100 kg/m^3. Together, these results suggest structural alterations in the skin epithelium at densities ≥100 kg/m^3.

In the specific water flow experiment, four different water flow levels were established (0.2, 0.3, 0.4 and 0.5 l/kg/min) while the fish density was kept constant at 75 kg/m^3. After eight weeks, transcription of *mucin-like 2* and *-5ac*, *inducible nitric oxide synthase*, *lysozyme* and *cathepsin B* and *-L* increased in skin samples from fish reared in tanks with a specific water flow of ≤0.3 l/kg/min. Increased transcription of these genes implies activation of stress and immune responses in skin at low specific water flow.

Results from this study suggests that skin is a sensitive organ for environmental changes, and suggests several molecular indicators that may be valuable in predicting the effects of varying rearing conditions on skin health. Further validation through long-term studies, combined with other health parameters is required for practical recommendations regarding critical fish density and water flow for optimal fish health and performance in semi-closed production systems.

Statement of relevance: This paper is of high importance for the industry as it investigates the effects of fish density and specific water flow on skin properties in semi-closed-containment-systems in Atlantic salmon post-smolt production. Increased fish densities and reduced specific water flow can increase production profitability. Thus, biological limits related to these two factors are important to identify to avoid reduced animal welfare.

1. Introduction

Low-cost open cages are the predominant type of cage used in salmon culture today. However, there are concerns related to this technology in regards to increased sea lice (*Lepeophtheirus salmonis*) pressure, escapes, nutrient discharge and fish mortalities (Gullestad et al., 2011). This has prompted several initiatives for testing semi-closed-containment technologies (S-CCS) in sea and closed-containment systems (CCS) in land-based facilities (Iversen et al., 2013). In both S-CCS and CCS, the species are separated from the surroundings by a physical hindrance. In S-CCS, water is exchanged from a natural waterway, whereas in CCS the water is treated and recycled.

In Norway, production of post-smolts up to 1 kg in size in CCS was permitted from 2011. However, since these systems carry with them

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high investment- and running costs, a high production intensity is required (Iversen et al., 2013; Terjesen et al., 2013). If the CCS technology is going to be cost-effective, densities must be greater than the current Norwegian legislation that limits fish densities in sea cages to 25 kg/m^3. Reduced water flow is a potential means to improve cost-efficiency in CCS. Existing recommendations from the Norwegian Food Safety Authorities suggest that water flow in closed facilities should be kept at minimum 0.3 l/kg/min (Rosten et al., 2004). Thus, research-based limits for maximum density and minimum specific water flow for Atlantic salmon (*Salmo salar* L.) post-smolts reared in CCS are needed.

Skin is the first defense barrier, being metabolically active and able to rapidly adapt to changes in the external environment. Thus, fish skin plays an important role in host defense, protection and preservation (Ángeles Esteban, 2012), and represents an important target tissue for evaluating welfare and health of farmed fish. Skin health depends upon several factors such as physical strength, ability of wound healing and resistance to pathogens (Esteban, 2012). Structurally, fish skin consists of three layers: the epidermis, dermis and hypodermis. The epidermis is the outermost layer where the majority of cells are epithelial cells and the minority are mucous cells (Elliott, 2011). The epithelial cells on the skin surface are linked with tight junctions, creating a physical barrier against the external environment, with claudins being one of the most important proteins (Gunzel and Fromm, 2012; Günzel and Yu, 2013). The tight junctions between epithelial cells act as a selective permeable barrier that regulate the movement of solutes between fluid compartments, thus they are important determinants of ion selectivity and general permeability of the epithelia (Chasiotis et al., 2012; Kolosov et al., 2013).

Mucous cells are differentiated epithelial cells that produce large glycoproteins (mucins), which are secreted onto the skin surface where they form the mucus layer. Several studies have reported that the number and size of mucous cells are affected by stressors such as pathogens, low pH and high concentrations of nitrate and aluminum (Ledy et al., 2003; Van Der Marel et al., 2010; Vatsos et al., 2010; Zuchelkowski et al., 1981). In addition to mucins, mucus also contains antibacterial peptides, immunoglobulins and enzymes. Lysozyme is one of the enzymes found in the mucus layer and its antibacterial properties cleave the 1,4-beta-linkages in the bacteria cell wall, thus playing a possible part of the innate immune system in fish (Esteban, 2012). Cathepsins are a large family of proteases that participate in protein degradation in lysosomes, endosomes as well as in cytosol and the nucleus. They are involved in a wide range of physiological processes in mammals and some of the reported functions are antigen processing, bone resorption and protein turnover (Brix and Stöcker, 2013; Colbert et al., 2009). Previous studies on enzymatic reactions associated with stress in Atlantic salmon demonstrate increased protease, lysozyme and cathepsin activity after prolonged or severe stress, but not at low stress levels (Easy and Ross, 2010; Ross et al., 2000). Another immune relevant protein is inducible nitric oxide synthase that produces nitric oxide through enzymatic oxidation of L-arginine. Nitric oxide is involved as a regulator and effector molecule in biological functions such as the maintenance of homeostasis, and also serving as an effector molecule in the immune system (Aktan, 2004; Thomas et al., 2015). Further, nitric oxide is also involved in adaptation to various stressors such as parasite infections (Gonzalez et al., 2007; Lindenstrøm et al., 2004), desiccation (Choudhury and Saha, 2012a) and high concentrations of ammonia (Choudhury and Saha, 2012b). Matrix metalloproteinases are a family of endopeptidase degrading a wide range of extracellular matrixes. One of the most studied metalloproteinases in fish is matrix metalloproteinase 9, which plays an in important role in wound healing processes during the inflammation and remodeling phase (Schmidt et al., 2016; Skugor et al., 2008; Sutherland et al., 2014).

Although a number of proteins are described and cellular functions are characterised, little is known about the salmon skin and how the external environment affects its composition and robustness. The aim of the present study was to investigate the effect of fish density and specific water flow on skin health in Atlantic salmon post-smolts reared in flow-through systems with full salinity, simulating the conditions in S-CCS at sea. Fluorescence staining of the epithelial cell surface was used to evaluate whether high fish densities and low specific water flow affect the amount of mucus, number of mucous cells or causes damage to the epithelial cell surface. In order to ensure correct validation of the results, the fluorescence staining was combined with traditional transcription analyses of genes known to be affected in skin during various stress conditions.

2. Materials and methods

2.1. Fish experiments, feeding management and sample collection

2.1.1. Fish stock and rearing conditions

Briefly, the fish used in this study were out-of-season smolts from the hatchery Lerøy Vest, Flateråker, in western Norway. First feeding started in early February 2012 under constant light and in heated water (12–14 °C). Between early May and early October the fish were maintained indoors in a green rearing tank (volume: 70 m^3) at constant light and water temperature (12 °C). All fish were fed a commercial dry diet (EWOS, Oslo, Norway) according to temperature and fish size. A photoperiod regime known to stimulate parr-smolt transition was initiated in the beginning of August (Handeland and Stefansson, 2001). This treatment included a decrease in day-length from LD24:0 to LD12:12 for five weeks followed by another four weeks on LD24:0. On October 8th, all fish showed normal morphological signs of smoltification, including silvery scales, dark fin margins, low condition factor and high gill Na+, K+ -ATPase activity.

2.1.2. Experimental design, fish density

The study was carried out at the Industrial Laboratory (ILAB), Bergen Norway, between October 10th and December 20th, 2012. On October 10th, 3750 smolts (mean weight (SEM) 115.0 g ± 13.6, mean length (SEM) 22.2 cm ± 1.4) were transported from the hatchery (Flateråker) to ILAB and distributed randomly among ten 1 m^2 square fiberglass tanks (500 l) with fish density as the experimental parameter (25.7, 50.1, 75.0, 100.8 and 125.2 kg/m^3, referred to as 25, 50, 75, 100 and 125 kg fish/m^3). Each treatment was conducted in duplicate tanks. In the period from the 16th to the 18th of October, the fresh water in each tank was gradually replaced with seawater; i.e. from 0 to 17‰ on October16th, from 17‰ to 25‰ on October 17th and from 25‰ to full strength seawater (34‰) on October 18th. Following transfer to seawater, the fish were exposed to a simulated natural light regime (60°25′N). The experimental period started on October 24th lasting till December 20th. In all groups, specific water flow was kept at 0.6 l/kg/min and temperature at 9.3 °C. Both temperature and oxygen saturation were measured daily (YSI 550, Xylem Inc., Yellow Springs, USA) in the outlet water of each tank, and pH was measured every week. The oxygen level in the outlet water was kept higher than 80% through oxygenation in the header tanks. All treatments were fed a commercial freshwater dry diet (Smolt 30, 2.8 mm, Ewos Norway) in 10% excess with automatic feeders daily between 09.00 and 10.00 and 15.00–16.00 throughout the study. A freshwater feed was used to reduce the sinking rate of the pellets increasing the availability time of the feed, thus minimizing the density dependent effect of feeding.

2.1.3. Experimental design, specific water flow

This study was carried out at the same time, in the same facilities, with the same fish material and with the same feed and water monitoring as described above. In this study fish were fed with an automatic feeder daily between 09.00 and 16.00. On October 10th 2012, 2500 smolts (mean weight (SEM) = 113.6 g ± 11.8, mean length (SEM) = 22.0 cm ± 0.99) were randomly distributed among eight 1 m^2 square fiberglass tanks (500 l, stocking density 75.0 kg/m^3) each with a specific

Table 1
Water quality parameters from the specific flow experiment (n = 2 tanks). Average values (±SE) are shown in the table.

Specific water flow (l/kg/min)	0.5	0.4	0.3	0.2
Water flow (l/min)	7.5	11.25	15	18.75
Tank exchange rate (min)	26.6	33.3	44.4	66.6
Temperature (°C)	9.3 ± 0.01	9.3 ± 0.01	9.3 ± 0.01	9.3 ± 0.01
pH	7.46 ± 0.05	7.37 ± 0.04	7.19 ± 0.05	6.9 ± 0.05
Carbon dioxide (mg/l)	4.79 ± 0.62	5.60 ± 0.48	8.6 ± 0.88	15.74 ± 1.83
Total ammonia nitrogen (mg/l)	0.36 ± 0.05	0.35 ± 0.05	0.48 ± 0.07	0.76 ± 0.11

sea water flow of 0.6 l/kg/min. The experimental treatments were established on October 24th and included four different specific water flow levels: 0.2, 0.3, 0.4 and 0.5 l/kg/min, each treatment was conducted in two replicate tanks. Water velocity in each tank was kept stable and equal by adjusting the angle on the inlet water pipe. Water quality parameters were measured in the outlet of each tank over the eight week experimental period (Table 1). The stocking density was kept at 75 kg/m^3 throughout the experimental period by removing the biomass gain from each tank every second week.

2.1.4. Sampling

Samples (n = 12 per treatment) were collected at the final sampling after eight weeks for both the fish density and specific water flow experiments. All individuals were fasted 24 h prior to sampling and anesthetized with 200 mg/l MS-222, a procedure avoiding any physical contact with the skin area from where the samples were taken. Skin samples were collected from a standardized 1 cm^2 area behind the dorsal fin and above the lateral line. Samples for gene expression analyses were frozen directly in liquid nitrogen and transferred to −80 °C for storage. The skin samples were fixed in 4% PFA overnight and then washed in 1 × PBST, before stepwise dehydration to 70% ethanol and transferred to −20 °C for storage.

2.2. Whole-mount skin staining

Before staining, the samples were rehydrated in decreasing ethanol concentrations and then permeabilized in 1 × PBST (phosphate buffered saline with 0.05% Tween-20) with 0.5% Triton ×100 for 30 min. Concanavalin A with Alexa Fluor® 647 Conjugate (Thermo Fisher Scientific Inc., Waltham, USA.) was applied for staining carbohydrates in the epithelial cell membrane with α-mannopyranosyl and α-glucopyranosyl residues. Wheat germ agglutinin (Thermo Fisher Scientific Inc.) with Alexa Fluor® 594 conjugate (Thermo Fisher Scientific Inc.) was applied for staining of cell membranes, mucus and mucous cells. Nuclei were stained using 4′, 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific Inc.). All stains were diluted in PBST at concentrations described by the manufacturer. After 30 min incubation and repeated washing in 1 × PBST, tissue samples were cleared in increasing glycerol concentrations to 99% before microscopy. For each tissue sample, three image stacks from representative areas on the middle of a scale were captured. All image stacks were batch deconvolved with Zeiss ZEN Blue software (Zeiss International) using optimal algorithm parameters for skin samples. Extended focus images were created from each z-stack and then scored blindly by two independent researchers.

Whole mount skin samples from 12 fish (n = 3 pictures per fish) were scored 0–3 for epithelial cell morphology, mucous cells and mucus amount. The epithelial cell morphology scored 0 represented the poorest cell morphology with damaged epithelial surface and the lack of cell-cell contact, or a very poor connection between neighboring epithelial cells. Samples scored 1 had areas devoid of epithelial cells and the remaining cells featured inferior contact with their neighbors. Samples scored 2 had complete epidermal layer, though cell-cell adherence were not as tight as the best scoring samples. A score of 3 represented good epithelial morphology and integrity, meaning a smooth surface consisting of a highly structured cell–cell contact. The number of mucous cells was evaluated similarly, where score 0 represented absence of mucous cells and score 3 high density of mucous cells, respectively. The amount of mucus inside each mucous cell was also evaluated, where a score of 0 represented low mucus content and 3 represented high mucus content.

2.3. RNA extraction

Tissue samples for RT-qPCR were stored at −80 °C prior to RNA extraction. Frozen samples of skin (0.5 × 0.5 cm) were transferred directly to 1 ml chilled TRIzol (Thermo Fisher Scientific Inc., Waltham, MA, USA) in 2 ml tubes with screw caps (Precellys®24, Bertin Technologies, Orléans, France). Two 2.8 mm zirchonium oxide beads (Precellys®24) were added to each tube and the tissue was homogenized in a Precellys®24 homogenizer for two times 25 s. at 5000 rpm with a pause of 5 s. between rounds.

RNA was extracted from the homogenized tissues using PureLink™ Pro 96 well purification kit (Thermo Fisher Scientific Inc.) with on-column-DNase (Qiagen, MD, USA) digestion according to the protocol for TRIzol-homogenized samples. The concentration of extracted total RNA was measured with a NanoDrop 1000 Spectrometer (Thermo Fisher Scientific Inc.).

2.4. Quantitative real-time PCR

Synthesis of cDNA was performed on 500 ng RNA with SuperScript® VILO™ Master Mix and SuperScript® VILO cDNA Synthesis Kit (Thermo Fisher Scientific Inc.) according to the manufactures instructions. Oligonucleotide primers were designed with the program Primer3 (v.0.4.0) and purchased from Thermo Fisher Scientific Inc. (Table 2). Amplicon size was set to 80–160 and melting temperature to 59–61 °C.

Table 2
Forward and reverse primers for RT-qPCR.

Gene name	Accession number		Primer sequence
Claudin 10	BK006391	F	ATCAAGGTGGCCTGGTACTG
		R	GACCAGAGCACAGGGAAGTC
Cathepsin L	NM_001146546.1	F	CCGGATACACACCTGGCTAC
		R	ACCCTCTACAGGCCCATTCT
Cathepsin B	NM_001140522.1	F	CCGGATACACACCTGGCTAC
		R	ACCCTCTACAGGCCCATTCT
cathepsin D	BT043515.1	F	CCATGCCTGACATCACATTC
		R	CCACTCAGGCAGATGGTCTT
Lysozyme	NM_001146413	F	TGGGAGGAGTTTCTGCTGTT
		R	ATCATGCTTGCTGCTGTTGA
Matrix metalloproteinase 9	NM_001140457.1	F	AGTCTACGGTAGCAGCAATGAAGGC
		R	CGTCAAAGGTCTGGTAGGAGCGTAT
Inducible nitric oxide synthase	AF088999.1	F	GCTAAACTGTGCCTTCAACTCCA
		R	CTCCATTCCCAAAGGTGCTAGTTA
Mucin-like 5ac	JT819124.1	F	AGGCGTCCTTGTCCAAATAA
		R	CCTCTGGAAACTGGATGGTC
Mucin-like 2	JT815394.1	F	ACCACCCTGAACCATCAGTC
		R	CTCCTTCAACATCGCATCAA
Reference genes			
Elongation factor 1 alfa	BT072490.1	F	CACCACCGGCCATCTGATCTACAA
		R	TCAGCAGCCTCCTTCTCGAACTTC
18S rRNA	AJ427629	F	GCCCTATCAACTTTCGATGGTAC
		R	TTTGGATGTGGTAGCCGTTTCTC

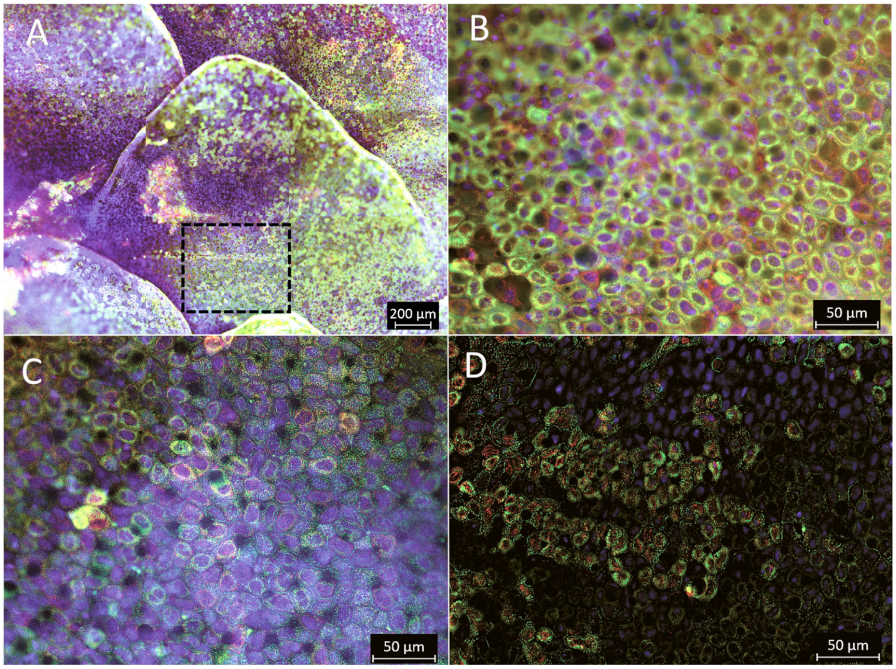

Fig. 1. Examples of fluorescence staining of whole-mount skin samples from representative individuals from the fish density experiment. Red fluorescence is ConA binding to lectins, green fluorescence is WGA binding to cell membrane and mucous cells and blue fluorescence is nuclear staining with DAPI. A) Overview picture of whole-mount skin sample, dotted square show standardized analysis area. Note the overlapping scales and differences in fluorescence intensity different areas of the tissue. Higher magnification of skin from representative fish reared at B) 25 kg/m^3, C) 50 kg/m^3 and D) 125 kg/m^3 respectively.

Quantitative real time PCR (RT-qPCR) was conducted using 2× SYBR® Green Master Mix (Roche Diagnostics, Mannheim, Germany) in an optimized 12 µl reaction volume, using 5 µl of 1:10 diluted cDNA, and primer concentrations of 0.42 µM. PCR reactions were prepared manually and run in duplicates in 96-well optical plates on a LightCycler 480 (Roche Diagnostics) with the following conditions: 95 °C for 5 min (pre-incubation), 95 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s (amplification, 45 cycles) and continuous increase from 65 °C to 97 °C with standard ramp rate (melting curve). Quantification cycle (Cq) values were calculated using the second derivative method. For evaluation of the results, the mean of duplicates was used. Duplicate measurements that differed >0.5 Cq values were removed and reanalyzed.

Relative expression ratios of test samples versus the average of the reference sample were calculated according to the Pfaffl method (Pfaffl, 2001). Elongation factor 1α (GenBank ID: BT072490.1) was used as reference gene (Jorgensen et al., 2006). The efficiency of the qPCR reactions were estimated for all primer pairs by six times 1:5 dilution series of a cDNA mix of all used samples. The efficiency values were estimated by using the LightCycler® 480 Software (version 1.5.0.39). All measured efficiencies were between 1.9805 and 1.999.

2.5. Data analyses and statistics

Statistical analyses were performed with R (www.r-project.org/, version 3.1.0). Gene expression data (relative fold changes) were $\log_2$ transformed for statistical tests and analyzed by Levene's test (Rcmdr package v2.0–4) for homoscedasticity. Subsequently, ANOVA was performed to identify significant differences between groups (R stats package v3.1.0). For ANOVA p-values < 0.05, a post hoc pairwise *t*-test with p-value correction according to Holm was performed (stats package) to detect which groups differ significantly from each other. In case of comparison of two groups, two-sample *t*-tests were used. p-Values < 0.05 were considered as significant. Whole-tissue staining score data were analyzed by Kruskal-Wallis rank test (stats package) and Wilcoxon rank tests (stats package). Data are represented as mean values ± S.E.M, unless otherwise is indicated.

3. Results

3.1. Fish density

3.1.1. High fish density affects epithelial cell morphology

Microscopy analyses of fluorescence stained whole-mount skin samples were conducted to visualize changes in epithelial cell morphology, number of mucous cells and mucus production correlating to fish density (Fig. 1). Fish reared at low fish density (25 kg/m^3) had the overall best epithelial cell morphology among the tested densities (Table 3). In these samples, the epithelial cells formed a continuous carpet of tightly connected cells, resulting in the highest epithelial cell morphology score (2.83 ± 0.11). Among fish reared at the highest density (125 kg/m^3) a significant deterioration in epithelial cell morphology was observed (2.08 ± 0.18), revealing poor cell-cell contact, or in some samples large areas devoid of epithelial cells. No significant differences in epithelial cell morphology were found for the fish densities 75 kg/m^3 and 100 kg/m^3. Notably, the samples from the 50 kg/m^3 treatment had distorted cell-cell contact and had the overall lowest epithelial cell morphology score (1.67 ± 0.27). No significant differences were found in the number of mucous cells or amount of mucus content in the mucous cells within the different density groups.

Table 3
Effects of fish density on epithelial cell morphology, number of mucous cells and mucus content. Skin samples from fish (n = 12) at each density were fluorescence stained and scored based on a standard scoring system. Mean score with ±standard error are shown in the table. Significant differences were marked with bold text. Group differences were marked with small type letters. Groups that do not share a letter were significantly different from each other.

Density (kg/m^3)	25	50	75	100	125
Epithelial cell morphology	**2.83 ± 0.11**^{a}	**1.67 ± 0.27**^{b}	**2.25 ± 0.21**^{ab}	**2.67 ± 0.14**^{ab}	**2.08 ± 0.18**^{b}
Mucous cells	2.67 ± 0.22	2.75 ± 0.17	2.67 ± 0.18	2.25 ± 0.27	2.08 ± 0.22
Mucus	1.5 ± 0.4	1.67 ± 0.34	1.33 ± 0.41	1.25 ± 0.38	0.75 ± 0.29

3.1.2. Fish density alters skin gene expression

To investigate whether high fish densities cause transcriptional changes in genes involved in mucus production, barrier and immune functions RT-qPCR was conducted on several genes known to be involved in these processes. *Cathepsin B, -L and -D* were all significantly up-regulated at 125 kg/m^3 compared to all the other density groups (Fig. 2A, B, C). Transcription levels of *matrix metalloproteinase 9* were significantly higher at both 100 and 125 kg/m^3 compared to the other density groups (Fig. 2G). *Claudin 10* was significantly up-regulated at 125 kg/m^3 compared to the 25, 50 and 75 kg/m^3 groups (Fig. 2D). *Mucin-like 2* was significantly ($p < 0.05$) up-regulated at 125 kg/m^3 compared to 25, 75 and 100 kg/m^3 (Fig. 2H). However, no significant

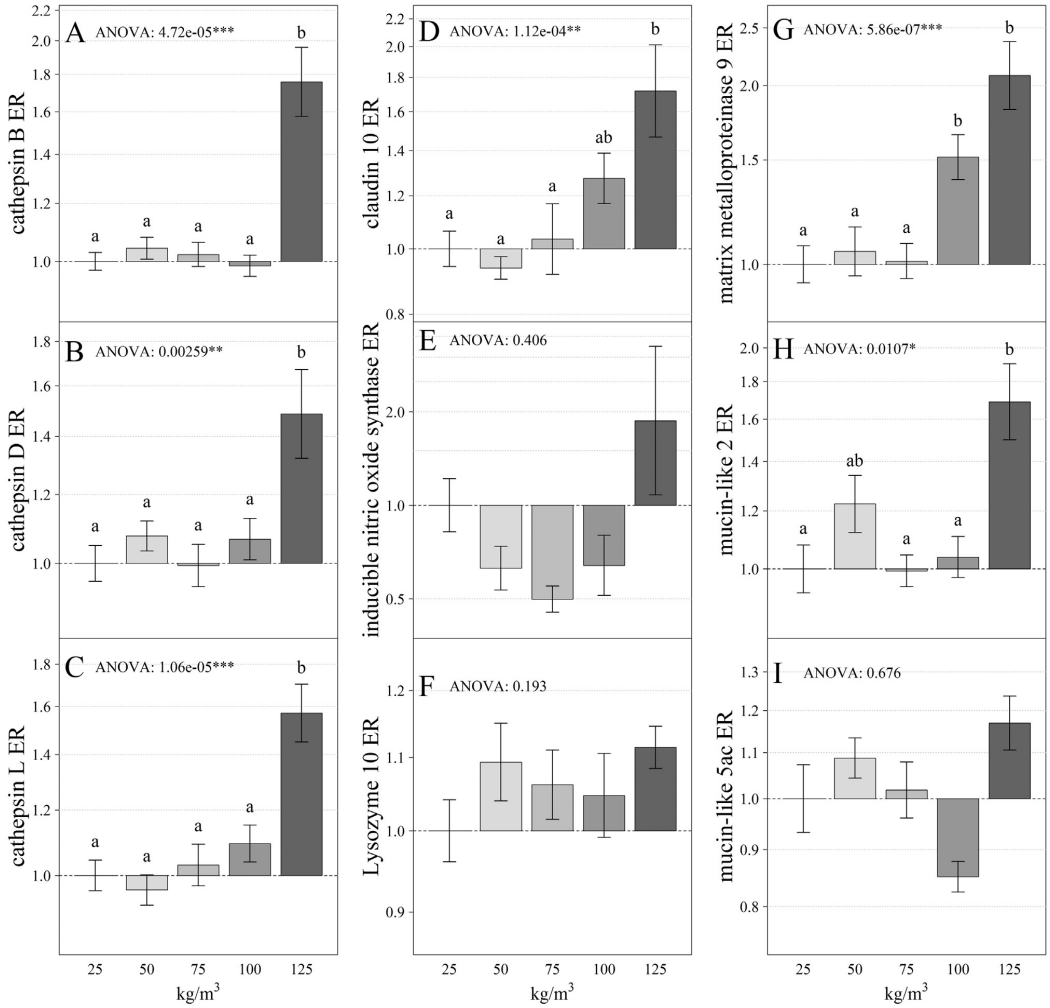

Fig. 2. Effects of fish densities on expression of target genes analyzed by real-time qPCR. Bars show mean gene expression ratio (with ±standard error) relative to the mean expression of the lowest density group (25 kg/m^3). ANOVA p-values are indicated in the plot. In case of ANOVA $p < 0.05$, Tukey post hoc tests were calculated. Groups which do not share a lower-case letter were significantly different from each other ($p < 0.05$). A) *cathepsin B* B) *cathepsin D* C) *cathepsin L* D) *claudin 10* E) *inducible nitric oxide synthase* F) *lysozyme* G) *matrix metalloproteinase 9* H) *mucin-like 2* I) *mucin-like 5ac.*

Table 4
Effects of fish density on epithelial cell morphology, number of mucous cells and mucus content. Skin samples from fish (n = 12) at each density were fluorescence stained and scored based on a standard scoring system. Mean score with ± standard error are shown in the table. No significant differences were found.

Flow (kg/l/min)	0.2	0.3	0.4	0.5
Epithelial cell morphology	1.83 ± 0.2	1.92 ± 0.28	2.18 ± 0.27	2.33 ± 0.22
Mucous cells	2.33 ± 0.25	1.92 ± 0.3	2.09 ± 0.23	2.42 ± 0.22
Mucus	1.5 ± 0.34	0.92 ± 0.32	1.55 ± 0.33	1.33 ± 0.38

difference in *mucin-like 2* gene expression was found between the highest density group and 50 kg/m^3.

3.2. Specific water flow

3.2.1. No effect of specific water flow on epithelial cell morphology

To investigate whether different levels of specific water flow cause structural alterations in the epithelial cell morphology, changes in mucous cell number or mucus amount, microscopy analyses of fluorescence stained whole-mount skin samples were conducted. No significant differences were found in epithelial cell morphology, number of mucous cells or mucus content (Table 4).

3.2.2. Specific water flow alters skin gene expression

To investigate whether different water flow levels cause transcriptional changes in genes involved in mucus production, barrier and immune functions, RT-qPCR was conducted on several genes known to be involved in these processes. RT-qPCR analysis showed overall higher transcription of investigated genes in the two groups with the lowest specific water flow compared to the two groups with higher specific water flow (Fig. 3). There was a clear separation in expression profiles between 0.3 and 0.4 l/kg/min, hence the groups with the lowest specific flow (0.2 and 0.3 l/kg/min) and the highest specific flow levels (0.4 and 0.5 l/kg/min) were pooled. After the pooling the mucin genes *mucin-like 2* and *mucin-like 5ac* showed significantly increased relative gene transcription in the 0.2–0.3 l/kg/min group compared to 0.4–0.5 l/kg/min (Fig. 3H, I). Correspondingly, an increased relative gene transcription was found for *cathepsins B, D* and *L* (Fig. 3A, B, C), *inducible nitric oxide synthase* (Fig. 3E) and *lysozyme* (Fig. 3F) in the 0.2–0.3 l/kg/min group compared to 0.4–0.5 l/kg/min group.

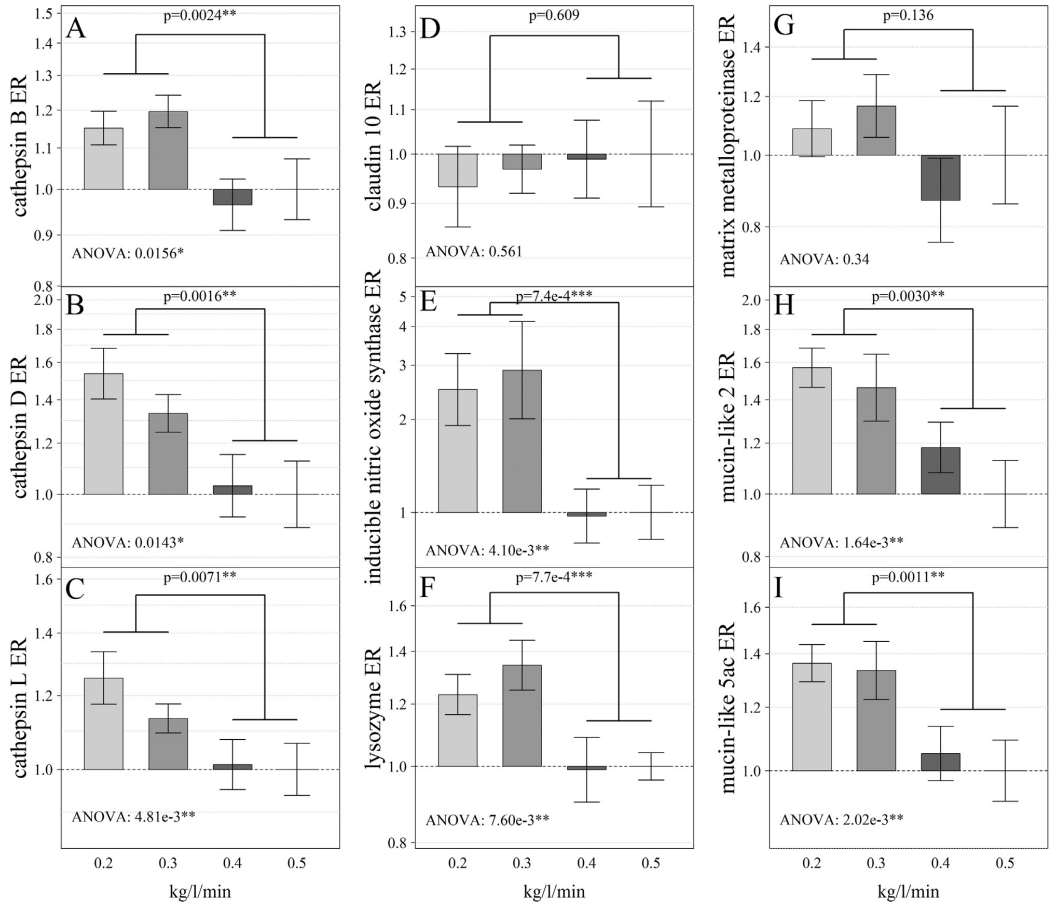

Fig. 3. Effects of specific water flow on selected genes analyzed with real-time qPCR. Expression ratio (ER) of genes relative to highest flow group (0.5 kg/m^3) as measured in skin; A) *cathepsin B* B) *cathepsin D* C) *cathepsin L* D) *claudin 10* E) *inducible nitric oxide synthase* F) *lysozyme* G) *matrix metalloproteinase 9* H) *mucin-like 2* I) *mucin-like 5ac.* Bars indicate the mean and error bars the standard error of mean. ANOVA p-values for the four groups are indicated in the plot. Significant differences between 0.2 and 0.3 kg/l/min compared to 0.4–0.5 kg/l/min (*t*-tests) are indicated in the figure with p-value.

4. Discussion

The two experiments described in this study were designed to simulate conditions in S-CCS at sea, testing five fish densities and four specific water flow levels that are relevant for the salmon farming industry (Thorarensen and Farrell, 2011). In the density experiment, microscopic examination of fluorescence stained whole-mount skin samples demonstrated significant differences in epithelial cell morphology, with increased spacing between epithelial cells at fish densities of 50 kg/m^3 and 125 kg/m^3. Gene expression analysis revealed increased transcription of several genes involved in immunity and repair mechanisms in the skin at fish densities ≥100 kg/m^3. In the specific water flow experiment, gene transcription analysis revealed significantly higher transcription of genes involved in cellular stress and immunity at water flow ≤0.3 l/kg/min compared to specific water flow ≥0.4 l/kg/min.

Transcription of nine different genes was evaluated to investigate the effect of increased fish density and reduced specific water flow on skin health. Genes in the *cathepsin* and *mucin* family were the only genes with increased transcription in both experiments.

Cathepsins were chosen as markers for cellular turnover and protein remodeling in the skin. Transcription of *cathepsin B, -D* and *-L* increased significantly at a density of 125 kg/m^3. Increased transcription of *cathepsin B, -D* and *-L* was also detected at a water flow rate of 0.2–0.3 l/kg/min. Previous studies have demonstrated that cysteine proteinases such as cathepsin B and -L are commonly expressed in the skin of Japanese eel (*Anguilla japonica*), further environmental stimuli such as thermal stress and external bacterial exposure enhances the proteolytic activity in epidermis, probably through increased activity of cathepsins (Aranishi et al., 1998). Cortisol may be a mediator for increased peripheral proteolysis in fishes (Mommsen et al., 1999). The increased transcription of cathepsins in skin at a fish density of 125 kg/m^3 and water flow rate of 0.2–0.3 l/kg/min demonstrate that these genes respond to different environmental stimuli. Both high fish densities and reduced specific water flow increased the transcription of several cathepsins, indicating a need for increased proteolytic activity in the skin under these conditions.

Two mucin genes were chosen as markers for mucous cell activity and mucus production in Atlantic salmon skin. Transcription of *mucin-like 2* increased at a density of 125 kg/m^3 while transcription of *mucin-like 2* and *mucin-like 5ac* increased with decreasing water flow rate of 0.2–0.3 l/kg/min. At high fish densities, it is possible that the increased mucin transcription could be due to epithelial damage. Wounds have earlier been reported to increase transcription of mucin genes. In experimentally wounded common carp (*Cyprinus carpio*), transcription of *muc5b* increased not only in the wound but also as a general response in the skin mucosa (Przybylska-Diaz et al., 2013). At high fish densities, increased mucin transcription could therefore indicate a response to the observed deterioration in epithelial cell morphology. It is also possible that the increased mucin transcription could be due to changes in the water quality parameters; this accounts for both the density and specific water flow experiments. Due to the metabolism of the fish, carbon dioxide and ammonia levels will increase as the water exchange is reduced or biomass increased. Increased biomass and reduced specific water flow may also cause accumulation of particles and bacteria in the water. Several authors have previously demonstrated that different water quality parameters can affect the number of mucous cells. In sea bass (*Dicentrarchus labrax*) both high nitrate concentrations and low oxygen concentrations increased the number of mucous cells in the skin (Vatsos et al., 2010). Increased numbers of epidermal skin mucous cells were noted in brown bullhead catfish (*Ameiurus nebulosus*), following exposure to acid, (Zuchelkowski et al., 1981; Zuchelkowski et al., 1985), and water with increased bacterial load introduced changes in the skin mucosal response in common carp (Van Der Marel et al., 2010). The observed increase in mucin transcription in the present study may be due to changes in water quality parameters. In conclusion, both high fish densities and low specific water flow trigger mucin transcription which may indicate that the fish either adjust to changes in water quality parameters, or experience epithelial damage, or a combination of both. Further studies of the specific transcription pattern of more mucin genes during different rearing conditions are warranted as these will provide insight into mucosal protection. In the present study, no correlation was found between the number of mucous cells and mucus amount with the transcription of the mucin genes.

Five out of nine genes had increased transcription only in the density or the specific water flow experiment. High fish densities led to increased transcription of *claudin 10* and *matrix metalloproteinase 9* in Atlantic salmon skin. The tight junction protein *claudin 10* was used as a marker for cellular integrity and epithelial barrier function. Increased transcription of *claudin 10* at fish densities of 100 kg/m^3 and 125 kg/m^3 indicates a demand for proteins involved in maintaining the cellular integrity and barrier function in the skin. Many tight junction proteins have sealing functions and others like claudin 10 (Gunzel and Fromm, 2012) are channel-forming proteins involved in paracellular transport that feature selectivity for ions. In Atlantic salmon, *claudin 10* transcription in gill increased during smoltification and salt-water acclimation, suggesting that claudin 10 is involved in osmoregulation (Tipsmark et al., 2008). This is also true for euryhaline Japanese medaka (*Oryzias latipes*), where claudin 10 has been suggested to be involved in osmoregulation in gills and kidney (Bossus et al., 2015). Cortisol treatment of cultured gill epithelia from puffer fish (*Tetraodon nigroviridis*) dose-dependently altered transcription of selected claudins (Bui et al., 2010). Previous studies have suggested a relationship between decreased levels of selected claudin proteins and increased gill permeability in the gills of puffer fish (Bagherie-Lachidan et al., 2008). In the present study the increased transcription of *claudin 10* at 125 kg/m^3 may be due to epithelial damage as the epithelial cell morphology also decreased at this density. Conversely, there was no relationship between increased *claudin 10* transcription and poor epithelial cell morphology at 100 kg/m^3. Further, fish reared at 50 kg/m^3 had the poorest epithelial cell morphology, yet the lowest transcription of *claudin 10*. Together these results indicate that increased *claudin 10* transcription is not directly linked to epithelial cell damage, but may be linked to other mechanisms triggered by high fish densities.

Matrix metalloproteinase 9 was used as an indicator for activation of cellular stress responses and potential activation of innate immunity and extracellular matrix degradation. Transcription of *matrix metalloproteinase 9* increased in the density experiment at fish densities of both 100 and 125 kg/m^3. In common carp, *matrix metalloproteinase 9* is expressed in classical fish immune organs and in peritoneal and peripheral blood leucocytes, indicating a role of *matrix metalloproteinase 9* in immune responses (Chadzinska et al., 2008). In vitro stimulation of common carp phagocytes with lipopolysaccharides increased *matrix metalloproteinase 9* transcription (Chadzinska et al., 2008). Transcription profiles of m*atrix metalloproteinase 9* in common carp also indicate a role during the initial phase of inflammation and during the later phase of tissue remodeling (Chadzinska et al., 2008). In rainbow trout, increased transcription of *matrix metalloproteinase 9* have been linked to the early inflammatory stages in wound healing but not in later stages (Schmidt et al., 2013). In the present study, reduction in epithelial cell morphology at 125 kg/m^3 may explain the increased transcription of *matrix metalloproteinase 9*. However, no reduction was found in the epithelial cell morphology at 100 kg/m^3. As described previously, changes in water quality parameters due to increased fish densities may also explain the increased transcription of *matrix metalloproteinase 9*. In conclusion, the observed increase in *matrix metalloproteinase 9* transcription may indicate that the cells respond to changes in the rearing environment or that *matrix metalloproteinase 9* is sensitive to skin damage when histological changes in cell morphology are not yet observable.

In the specific water flow experiment, transcription of *inducible nitric oxide synthase* and *lysozyme* increased at a specific water flow of 0.2–0.3 l/kg/min. These genes were not affected by increasing fish densities.

Inducible nitric oxide synthase is often used as a marker for cellular stress responses and activation of innate immunity. With respect to nitric oxide production, it is known that nitric oxide synthase activity is induced in catfish leucocytes following experimental challenge with gram negative bacteria (Schoor and Plumb, 1994) and that stimulation of a goldfish macrophage cell line with lipopolysaccharides induces nitric oxide release (Neumann et al., 1995). Phagocytes from common carp produce huge amounts of nitric oxide after stimulation with lipopolysaccharides (Saeij et al., 2000) and transcription of *inducible nitric oxide synthase* in head kidney and gill tissue have been detected in rainbow trout challenged with bacteria (Laing et al., 1999). Thus, the observed increase in *inducible nitric oxide synthase* transcription is likely to be linked to an increased need for mucosal protection in the skin. However, the increased transcription of *lysozyme* may indicate activation of the innate immunity in the skin. Lysozyme is present in mucus, lymphoid tissue, plasma and other body fluids of freshwater and marine fish, thus it is an important defense molecule of the fish innate immune system (Saurabh and Sahoo, 2008). In rainbow trout, lysozyme activity can be dependent on the degree of stress, as well as the intensity, duration and type of stressor (Yildiz, 2006). Rainbow trout exposed to handling stress had increased lysozyme activity in plasma (Demers and Bayne, 1997). Enhanced serum lysozyme activity was also found in Atlantic salmon experimentally challenged with *Aeromonas salmonicida* infection (Møyner et al., 1993). Factors in the aquatic environment such as salinity, pH and suspended solids can also affect lysozyme in mucus from Atlantic salmon (Fast et al., 2002; Saurabh and Sahoo, 2008). Observed in this study, the increased transcription of *inducible nitric oxide synthase* and *lysozyme* at low specific water flow levels is likely due to changes in the water quality parameters, as described above.

In the present study, results from the fish density experiment on the fluorescence stained whole-mount skin samples demonstrated that the epithelial cell morphology score decreased at a fish density of 50 kg/m^3 and 125 kg/m^3. Conversely, no significant differences were found for fish densities of 25 kg/m^3, 75 kg/m^3 and 100 kg/m^3. Previous studies have investigated the effect of fish density on the growth of Atlantic salmon (Berg et al., 1996; Kjartansson et al., 1988; Soderberg et al., 1993), however none of these studies included molecular or histological evaluation of skin. Results from fish density studies are generally difficult to compare because they operate with different density groups, different density ranges and different stages in the fish's life history (Thorarensen and Farrell, 2011). Nevertheless, a review by Thorarensen and Farrell (2011) conclude that densities up to 80 kg/m^3 do not limit the growth and survival of Atlantic salmon post-smolts. Relevant to our observations on skin damage, fin erosion has been reported as a common problem when fish densities increase (Ellis et al., 2002). Previous studies on Atlantic salmon have found that densities above 22 kg/m^3 (in the range 9.7 to 34 kg/m^3) (Turnbull et al., 2005) can be associated with reduced fin conditions and fish reared at densities below 30 kg/m^3 have less pronounced fin damage (Jones et al., 2011). In the present study, the observed decrease in epithelial cell morphology at 50 kg/m^3 and 125 kg/m^3 could therefore be due to increased skin abrasion and dermal injuries. For the density of 125 kg/m^3, this is supported by the gene transcription data where in total six genes known to be involved in wound healing mechanisms had increased transcription (*cathepsin B-, L* and *D*, *matrix metalloproteinase 9, claudin 19* and *mucin-like2)*. However, there was no link between gene transcription and reduced epithelial cell morphology at 50 kg/m^3. Overall, there was no clear relationship between reduced epithelial cell morphology and increasing fish densities. This indicates that there could be other underlying mechanisms triggering increased gene transcription at high fish densities.

In the specific water flow experiment there was no association between epithelial cell morphology and flow rates. The reason for the reduction in epithelial cell morphology in the density experiment may be explained by skin abrasions caused by altered swimming pattern and behavior, which would be unlikely to occur at different specific water flow levels.

In conclusion, our results suggest impaired skin health at fish densities of 50 and 125 kg/m^3, implied from reduced epithelial cell morphology together with induced transcription of genes involved in barrier and epithelial repair functions, possibly due to suboptimal water quality and/or increased skin abrasion. A fish density at or above 100 kg/m^3 also resulted in increased transcription of *matrix metalloproteinase 9* and *claudin 10*, implying elevated cellular stress also at these densities. The range of specific water flow treatments affected neither epithelial cell morphology nor mucus integrity. However, water flow ≤0.3 l/kg/min caused increased transcription of genes involved in innate immunity and mucus production, possibly through changes in water quality parameters. In both experiments, the observed changes in gene expression may simply reflect that fish are coping with the specific stressor. Long-term studies in combination with other welfare indicators required to elucidate any detrimental effects.

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PLOS ONE

RESEARCH ARTICLE

Genome-wide analysis of Atlantic salmon (*Salmo salar*) mucin genes and their role as biomarkers

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Abstract

The aim of this study was to identify potential mucin genes in the Atlantic salmon genome and evaluate tissue-specific distribution and transcriptional regulation in response to aquaculture-relevant stress conditions in post-smolts. Seven secreted gel-forming mucin genes were identified based on several layers of evidence; annotation, transcription, phylogeny and domain structure. Two genes were annotated as *muc2* and five genes as *muc5*. The *muc2* genes were predominantly transcribed in the intestinal region while the different genes in the *muc5* family were mainly transcribed in either skin, gill or pyloric caeca. In order to investigate transcriptional regulation of mucins during stress conditions, two controlled experiments were conducted. In the first experiment, handling stress induced mucin transcription in the gill, while transcription decreased in the skin and intestine. In the second experiment, long term intensive rearing conditions (fish biomass ~125 kg/m^3) interrupted by additional confinement led to increased transcription of mucin genes in the skin at one, seven and fourteen days post-confinement.

1. Introduction

The mucus matrix that lines the epithelia of all the mucosal tissues has an important but poorly understood role in protection. The main constituent of the mucus matrix is the large gel-forming glycoproteins called mucins [1]. Most knowledge regarding mucins comes from studies in mammals. Intensive research on mammalian mucins is due to the involvement of mucins in intestinal protection [2], cancer [3] and diseases of the respiratory system [4].

In commercial aquaculture, fish are exposed to more stressful events than their wild relatives, which may make them more susceptible to diseases [5–7]. The route of infection is usually through the skin, gill or gastrointestinal regions, which are the main mucosal tissues. These tissues contain mucous producing cells which secret a protective mucus matrix that cover the epithelial surfaces. The matrix protects the surface both by its physical properties

study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

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and by acting like a medium, facilitating the action of defense molecules and other biologically active substances [8]. The number of mucous producing cells has been suggested to reflect the health status of the mucosal tissues [9]. Increased number of mucous cells in the skin have been seen in response to external stressors such as reduced pH [10, 11] high nitrate and low O_2 levels [12], aluminum exposure [13] and presence of pathogens [14]. Increased number of mucous cells are also reported in gills in relation to various stressful conditions such as presence of exotoxins [15] and changes in water quality [16]. Despite several publications related to mucous cell number and the protective role of the mucus layer, little knowledge exists about the mucin-encoding genes in fish. Recently, Perez-Sanchez et. al. (2013) demonstrated that intestinal mucin transcription responded to both diet and parasite infection, suggesting that mucins may be used as genetic markers for fish intestinal health.

In humans and higher vertebrates, more than twenty different mucin genes have so far been identified [2], while eleven different gel-forming mucins have been found in the model fish species Zebrafish (*Danio rerio*) [17]. The mucin proteins are separated into two functional classes: The secreted gel-forming mucins and the membrane bound mucins. The present study focuses on the gel-forming mucins.

The secreted gel-forming mucins are characterized by the presence of several domain structures; von Willebrand D (VWD), cysteine rich (C8) and trypsin inhibitor like cysteine rich (TIL) domains. These domains contribute to oligomerization of the mucin proteins through disulfide bond formation [18], which provides the gel-forming properties of mucus [19]. In addition, some mucins also have a C-terminal cysteine knot (Cys-Knot) domain, von Willebrand C (VWC) domain and the domain Mucin2_WxxW [17]. In humans, MUC2, MUC5AC and MUC5B all have the domain architecture (VWD-C8-TIL)-(VWD-C8-TIL)-(VWD-C8-TIL)-PTS-(VWD-C8-TIL) [17]. In addition, the secreted gel-forming mucins have long segments of highly repetitive sequences that are rich in proline, threonine and serine residues, referred to as the PTS-domain [20]. The number, length and amino acid sequence of the PTS domains varies among the mucins and these domains are poorly conserved among species [21]. The serine and threonine residues are sites for O-linked glycosylation which provides rigidity and solubility to the protein [18].

In Atlantic salmon, three isotigs have been predicted that exhibit homology to the mammalian mucins MUC2, MUC5AC and MUC5B; however due to short sequences, no definite identification was made in the published research [22]. As several authors have reported, the large size and the repetitive nature of the mucin genes makes their identification difficult [17]. In addition, several other non-mucin proteins also contain the VWD domain and the domain structure VWD-C8-TIL; confounding verification that the identified gene encodes an actual mucin [21].

In the current study, identification of putative mucin genes in the Atlantic salmon genome was based on several layers of evidence including domain architecture, phylogeny and transcription patterns from public RNAseq data. In addition, transcription patterns of the identified mucin genes were analyzed with quantitative real time PCR (qPCR) in mucosal tissues from healthy Atlantic salmon. Lastly, two experiments were conducted to investigate how the identified mucin genes respond to external stressors that are relevant in the commercial production of Atlantic salmon.

Materials and methods

Bioinformatics

Annotation and sequences. All sequences annotated as mucins were extracted from the Atlantic salmon genome (NCBI Reference Sequence Database (RefSeq) assembly accession:

GCF_000233375.1), in total 144 sequences. PFAM domains for all protein coding genes were identified by querying each predicted protein sequence against the Pfam-A.hmm database, using hmmscan [23] (version 3.1b). As the Pfam-A.hmm database does not contain PTS domains, the PTS domains were identified using an in house R-script according to [24]. In brief, a sliding window algorithm was used to identify regions containing $>$ 40% serine or threonine and $\geq$ 5% proline, using a window size of 100 amino acids. Sequences containing a domain structure of minimum VWD-C8-TIL were considered to be potential mucin candidates. Out of the 144 sequences 25 had this domain structure.

RNAseq data. A publicly available RNAseq dataset spanning 9 different tissues (Sequence Read Archive: PRJNA260929) [25], was used to evaluate transcription for all the identified potential mucin or mucin-like genes (144 genes). In total, 52 out of the 144 sequences were transcribed in one or more tissue.

Domains and phylogeny. The phylogenetic tree was built by aligning the conserved VWD domains. The sequences of the VWD domains, hereafter referred to as D1-D4 based on their relative position in the protein starting from the N-terminus, were used in the alignment. Public available mucin sequences from other species were downloaded from Ensemble [26], (S1 File). Protein sequences from all VWD domains were extracted and numbered in sequence from the N-terminus. The domain sequences were aligned using mafft (v7.215) [27]. The alignments were subsequently imported into R where the phylogenetic tree was built using a maximum likelihood model (*optim.pml* function implemented in the "phangorn" R package [28], using the WAG substitution model for AA[29]). Branch support was evaluated by bootstrap analysis based on 100 pseudoreplicates using the *bootstrap.pml* function [28]. An in-house R-script was used to build domain structures for the Muc2 and Muc5 families from the downloaded sequences.

Fish experiments and tissue collection

Tissue-specific transcription study. Post-smolts (SalmoBreed strain, Norway) were reared in 500 L square fiberglass tanks (25 kg/m^3) under standard conditions in a flow-through system with 32 ppt seawater at Nofima's Centre for Recirculation in Aquaculture station (Sunndalsøra, Norway) from May 2015. Temperature was 12˚C and O_2 saturation $>$ 80%, measured daily in the outlet water. Fish were fed Skretting Spirit Supreme 75 until July, when feed was changed to Ewos Opal 200. Fish were fed continuously from automatic feeders. On the 17th of August 2015 samples were collected from fish (n = 3) with an average body weight of 300 g. Samples were collected from skin (operculum, dorsal, ventral and caudal side of the body), fins (dorsal and caudal fin), eye, tongue, esophagus, stomach, intestine (anterior, middle and posterior parts), pyloric caeca, gallbladder and ovarium (Fig 1).

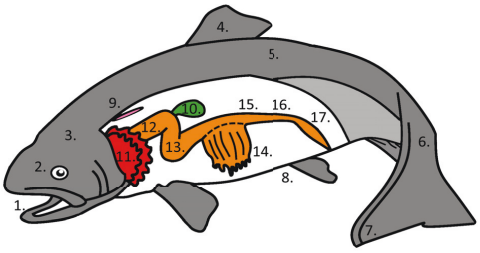

Fig 1. Tissue sections for mucin transcription analyses with quantitative real time PCR. 1. Eye 2. Tongue 3. Operculum 4. Dorsal fin 5. Dorsal skin 6. Caudal skin 7. Caudal fin 8. Ventral skin 9. Ovarium 10. Gall bladder 11. Gill 12. Esophagus 13. Stomach 14. Pyloric ceca 15. Anterior intestine 16. Middle intestine 17. Posterior intestine.

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Stress experiments. Two experiments were conducted for studies on mucin transcription following stress conditions; 1) acute short-term handling stress, 2) intensive rearing with high fish density followed by transient acute confinement stress.

Experiment 1: The same post-smolts were used as described above (tissue-specific transcription study). Control samples (n = 15) were collected prior to stress treatment. Fish were individually netted and exposed to air for 30s followed by 60s recovery in water that was continuously oxygenated (Tetratec APS 300, Southampton, UK) with O_2 levels > 95%. This procedure was repeated three times. The fish were then transferred to the original tank and samples from skin (dorsal side), gill and middle intestine were collected 3 h and 24 h post stress.

Experiment 2: This study was carried out at the Industrial Laboratory (ILAB, Bergen Norway) between October 4th and December 15th, 2014. Smolts (mean size 80 g) were distributed randomly among in 1 m^2 square fiberglass tanks (500 L). The fish were stocked at a density of 125 kg/m^3 in triplicate tanks. From the 4th to the 6th of October, the fresh water in each tank was gradually replaced with seawater (12˚C). Following transfer to full strength seawater, the fish were exposed to a simulated natural light regime (60°25'N). The fish were fed a commercial dry diet (EWOS, Oslo, Norway) in 10% excess between 09.00–10.00 and 15.00–16.00 throughout the study. Temperature and O_2 levels as described above. The experimental period started on the 28th of November and lasted until the 15th of December. On the 1st of December the fish were exposed to confinement stress by lowering the water level in the tanks to 20 cm for 30 min giving an effective density of 500 kg/m^3. Prior to sampling the fish were killed with an overdose of anesthetic (Finquel, Scanvac Årnes, Norway). Skin samples (dorsal side) were collected on the 28th November (time zero; average weight 103g ± 23) and the 2nd (day 1; 99g ± 14), 8th (day 7; 96g ± 19) and 15th (day 14; 96g ± 25) of December. All samples were snap frozen in liquid nitrogen and stored at -80˚C.

Ethical statement: This study was approved by the national ethics committee (the Norwegian Food Safety Authority) and carried out in strict accordance with the Norwegian animal welfare act (LOV-2009-06-19-97) and the regulation on the use of animals in research (FOR-2015-06-18-761).

Quantitative real-time RT-PCR (qPCR). Frozen skin sections were transferred to TRIzol (Thermo Fisher Scientific) and homogenized with 2,8 mm zirconium oxide beads (Precellys®24) in a Precellys®24 homogenizer (Precellys®24). RNA was extracted using PureLink™ Pro 96-well purification kit (Thermo Fisher Scientific) with on-column-DNase (Qiagen) digestion. RNA concentration and quality was measured with NanoDrop (Thermo Fisher Scientific). Synthesis of cDNA was performed on 500 ng RNA with SuperScript® VILO cDNA Synthesis Kit and Master Mix (Thermo Fisher Scientific). Primers were designed with Primer3 (v. 0.4.0) [30, 31] (Table 1). QPCR was performed in duplicates in 96-well optical plates on a LightCycler 480 (Roche Diagnostics). Each well had a final reaction volume of 12 μl, using 6 μl SYBR® Green Master Mix (Roche Diagnostics), 5 μl of 1:10 diluted cDNA and primers at a final concentration of 0.42 μM.

Quantification cycle (Ct) values were calculated using the second derivative method (stress experiments) or the fit-points method (tissue specific transcription study) in the LightCycler 480 software (version 1.5.0.39). Duplicates showing a Ct difference < 0.5 were removed from further analysis. The efficiency of the qPCR reactions were estimated for all primer pairs by five times 1:2 dilution series. Two reference genes were evaluated for stability using the web-based comprehensive tool RefFinder which integrates the computational programs geNorm, Normfinder, BestKeeper and the comparative delta-Ct method [32]. The most stable reference gene was *etif3* and this gene was used in the normalization procedure.

The specificity of the reactions was verified by analysis of the melting curves, electrophoresis and sequencing of the amplified PCR products. Prior to sequencing, fragments were

Table 1. Primers and general information.

RefSeq gene name	Symbol	Gene–ID	Main tissue of transcription	Chromosome	Forward primer	Reverse primer	AL/E[a]
muc5ac-like	*muc5ac.1*	XP_013982550.1	Skin	11	gacctgctctgtggaaggag	agcacggtgaattcagttcc	120/ 1.9
muc5ac-like[b]	*muc5ac.2*	XP_013981532.1	Gill	10	ttttctcagttgccgctttt	agtcggagcccataagaggt	92/ 1.8
muc5ac-like	*muc5ac.3*	XP_014031311.1	Pyloric ceca	26	-	-	
muc5ac-like[b]	*muc5ac.4*	XP_014037804.1	Gill	Scaffold	ttttctcagttgccgctttt	agtcggagcccataagaggt	92/ 1.8
muc5b-like	*muc5b*	XP_014031349.1	Skin	26	attaagagcgatgtcttcacagc	aagcacatgagtctctcacacaa	85/ 1.9
muc2-like[c]	*muc2.1*	XP_014025861.1	Intestine	23	gagtgggctctcagatccag	gatgatgcggacggtagttt	99/ 1.9
muc2-like[c]	*muc2.2*	XP_014040158.1	Intestine	Scaffold	gagtgggctctcagatccag	gatgatgcggacggtagttt	99/ 1.9
Reference genes							
Elongation factor 1 alfa	*elf1a*	AF321836			caccaccggccatctgatctacaa	tcagcagcctccttctcgaacttc	78/ 1.9
Eukaryotic translation initiation factor 3	*etif3*	DW542195			caggatgttgttgctggatggg	acccaactgggcaggtcaaga	102/ 1.9

[a]AL/E: cDNA amplicon length/Primer efficiency

[b] Primers bind *muc5ac.2* and *muc5ac.4*

[c] Primers bind *muc2.1* and *muc2.2*

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amplified with AmpliTac Gold® DNA polymerase (Applied Biosystems) and dNTPs (Promega) in a 10μl PCR reaction. ExoSap IT (Affymetrix) was used to remove excess primers and nucleotides according to the manufactures instructions. The sequencing reaction was run using a BigDye Terminator v.1.1 Cycle Kit (Applied Biosystems) and cleaned with a BigDye Xterminator Purification Kit (Applied Biosystems). Sanger sequencing was performed on 3130xl Genetic analyzer (Applied Biosystems) and sequences were analyzed with Sequence Scanner v2.0 (Applied Biosystems).

Data analysis. All data analysis were performed in R (www.r-project.org). In the tissue specific transcription study the delta Ct-values were averaged for each gene and tissue. The values were inverted and subsequently plotted as a heat map where the transcription in each tissue is presented relative to each other. Each square represents the average delta-Ct value of three individual animals (n = 2 for esophagus, stomach and ovarium).

Experiment 1: Significant changes in transcription were estimated by fitting a linear model to the delta-Ct values, separately for each gene and tissue. Groups showing a p-value < 0.05 were considered to be significantly different from the control group.

Experiment 2: Significant changes in transcription were estimated by fitting a linear model to the delta-Ct values for each gene. Groups showing a p-value < 0.05 were considered to be significantly different from day 0.

Results and discussion

We are just beginning to understand the complex nature of mucosal protection, a system which consists of both bioactive compounds and physical protection. The main constituents of the mucus matrix are the mucin proteins and the present study is the first comprehensive characterization of Atlantic salmon mucins, describing domain structure, phylogenetic relationship, tissue-specific

transcription and transcriptional regulation in response to stress conditions relevant for aquaculture production of salmonids. Seven unique mucins were identified as secreted gel-forming mucins in the Atlantic salmon reference genome, including two mucins annotated as *muc2* and five mucins annotated as *muc5*. In short, this study contributes to the understanding of mucosal protection which may provide a direct pathway to enhance the health and welfare of farmed fish.

Identification and structural characterization of putative mucin genes

Our search for Atlantic salmon mucin genes began with the identification of all the genes annotated as mucins in the recently released Atlantic salmon genome [25]. This approach identified 144 potential mucins (Fig 2A). Further, transcription profiles for the 144 genes were analyzed with a publicly available RNA-seq dataset [25]; from this it was evident that 52 genes were transcribed in one or more tissues, while the remaining 92 genes had very low or no detectable transcription (FPKM $< = 1$). The sequences of all the annotated mucins were then analysed for domain structure. This resulted in a total of 25 genes that contained the VWD-C8-TIL domain structure. Combining these three approaches, annotation, domain structure and transcription, seven mucin genes were identified, two genes in the *muc2* family and five genes in the *muc5* family (Fig 2A). In order to separate between the different mucin genes within the same family, a symbol was assigned to each of the genes (Table 1).

The domain structure of the seven putative mucin genes is presented together with the domain structure of mucins from other species (Fig 2B). Here we see that only Muc5b had the domain architecture of 3X(VWD-C8-TIL-VWC)-PTS-(VWD-C8-TIL), which is expected for the Muc5 family [17]. The remaining mucins lacked the PTS domain and one or more of the VWD domains. The PTS-domain is where the repetitive sequences are found, which often cause problems for sequencing and alignments [21]. In addition, PTS domains are only loosely defined and here we used the definition of a PTS domain as described in Lang et. al. 2004, meaning that there may be areas suitable for glycosylation in the sequence. Lastly, we examined the transcription pattern from the seven putative mucin genes (Fig 2C). As expected, the mucin genes were mainly transcribed in the mucosal tissues. The genes in the *muc2* family had a similar tissue distribution, being mainly transcribed in the foregut and pyloric caeca while the genes in the *muc5* family had a wider tissue distribution. *Muc5ac.1* and *muc5b* were mainly transcribed in the skin, *muc5ac.3* in pyloric caeca and *muc5ac.2* and *muc5ac.4* in the gill. These results match the profile of secreted gel-forming mucins in other species (discussed in the qPCR section below). In conclusion, seven putative mucin genes were identified using annotation, transcription and domain structure approaches. Both the transcription profile and the domain structure of these genes matches the profile of mucin genes in other species which supports our hypothesis that these genes are genuine mucins.

The major purpose of constructing the phylogenetic trees was to verify the classification of the predicted mucins. The phylogenetic tree was built based on the alignments of the VWD domains (Fig 3), as these domains represent the conserved areas in the genetic sequence [17]. In total, 19 Atlantic salmon VWD domains were aligned, corresponding to the number of the identified VWD domains found in the seven putative mucin sequences (Fig 2C). The Atlantic salmon domains were aligned with VWD domains both from fish species, and more distant related species such as mice and human. The Atlantic salmon mucins reflected the evolutionary relationships among species. Further, the tree clustered VWD domains from the Muc2 family together and the Muc5 family together, demonstrating a close interspecies relationship between these domains. Thus, we are quite certain that the seven putative mucin genes were annotated as the correct mucin variant.

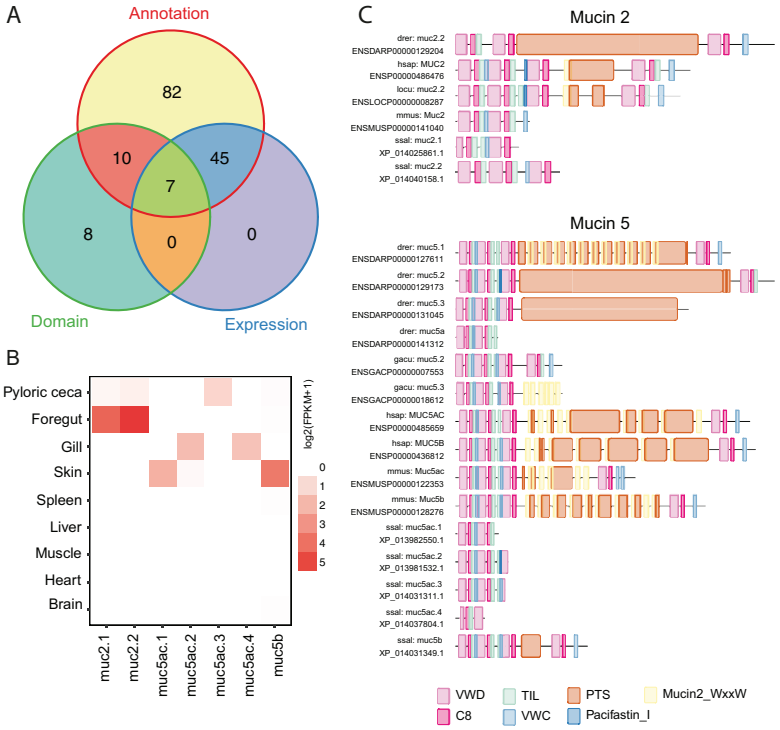

Fig 2. Identification of seven putative mucin genes, with annotation, transcription (public RNAseq data) and protein architecture. A According to RefSeq annotation, 144 genes were annotated as mucins, out of these 52 of were considered to be transcribed, whereas 25 genes had a minimum domain structure of VWD-C8-TIL. Seven mucin genes were found using the combination of these three approaches. **B** Protein architecture of the seven putative Atlantic salmon (*Salmo salar*, **ssal**), mucin genes. For comparison, the protein architecture of Muc2 and Muc5 from stickleback (*Gasterosteus aculeatus*, **gacu**), zebrafish (*Danio rerio*, **drer**), mouse (*Mus musculus*, **mmouse**), human (*Homo sapiens*, **hsap**) is also presented in the figure. **C** Transcription of the seven putative mucin genes in the tissues; Pyloric ceca, foregut, skin, pancreas, gill, spleen, liver, muscle heart and brain. The data is based on a public RNA-Seq data set. FPKM values as indicated in the figure.

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Lastly we looked at the relationship between VWD domains with similar N-terminal positions. The Atlantic salmon VWD domains from the Muc5 family and Muc2.2 clustered together with VWD domains with similar N-terminal positions. This suggests that VWD domains with similar N-terminal positions are related to each other, and that the order of the domains are conserved in the analysed species. Muc2.1 was an exception to this, as the D1 domain was more closely related to the D2 family, and the D2 domain was more closely related to the D3 family. An explanation for this may be because one N-terminal VWD domain was missing in the sequence (Fig 2C). Overall, this close relationship between VWD domains with similar N-terminal position suggests that the seven predicted Atlantic salmon mucin sequences were assembled in a correct way by the RefSeq genome assembly.

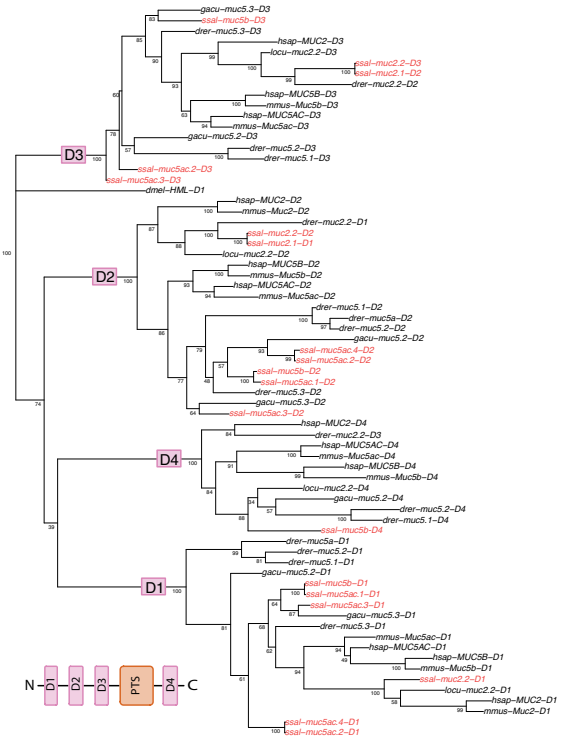

Fig 3. Phylogenetic tree of the identified Atlantic salmon mucins. The main branches represents clustering of the VWD domains, referred to as D1 to D4 based on their N-terminal position, as indicated in the insert figure. The Atlantic salmon (*Salmo salar*, **ssal**) VWD domains are highlighted in red, in total 19. In addition, VWD domains from several species are included in the tree, including stickleback (*Gasterosteus aculeatus*, **gacu**), spotted gar (*Lepisosteus* gacu, **locu**) zebrafish (*Danio rerio*, **drer**), mouse (*Mus musculus*, **mmouse**), human (*Homo sapiens*, **hsap**). The tree is rooted to the VWD domain found in Hemolectin of fruit fly (*Drosophila melanogaster*, **dmel**), a non-vertebrate mucin orthologue [21].

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Mucin transcription in mucosal tissues

Based on the seven putative mucin gene sequences, qPCR primers were designed to examine their transcription in a wide range of tissues from post-smolt salmon reared in seawater. Due to their highly repetitive nature and sequence similarity, it was difficult to obtain specific primers for all the seven mucin genes. Finally, four primer pairs were used in this study, two of these pairs bound to specific mucin sequences (*muc5ac.1* and m*uc5b*) and two pairs did not. One primer pair bound both copies of *muc2* (*muc2.1* and *muc2.2*) and the other primer pair bound to both *muc5ac.2* and *muc5ac.4*.

QPCR analysis of different tissue sections from the skin, fins and gastrointestinal tract showed that *muc2.1/2* was most strongly transcribed in the intestine with similar transcription levels between the anterior, middle and posterior parts (Fig 4). Transcription of *muc2.1/2* was also moderate in the pyloric caeca, low in the other analyzed organs and absent in the ovarium, tongue and caudal fin. Tissue transcription of the *muc5* family varied with the different genes.

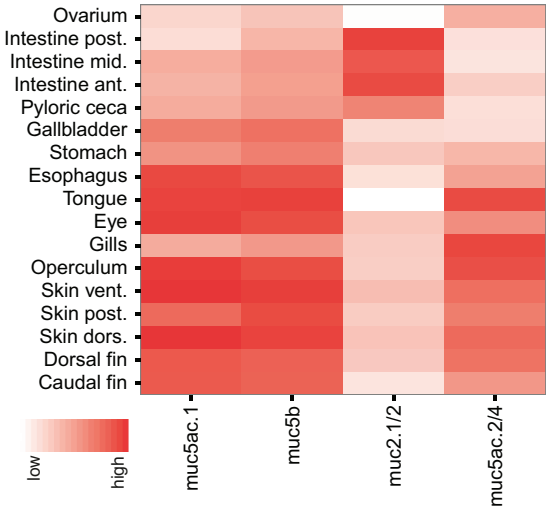

Fig 4. Mucin transcription levels in mucosal tissues, measured with qPCR. Transcription of *muc2.1/2*, *muc5ac.1*, *muc5ac.2/4 and* m*uc5b* in a set of different tissues. Each square represent the inverted mean of the delta-Ct-values n = 3 (n = 2 esophagus, stomach, ovarium).

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Muc5ac.1 and *muc5b* had similar transcription patterns, with high transcription levels in the skin, fins, eye, esophagus and tongue. This transcription pattern was different from *muc5ac.2/4* that were highly transcribed in the gill and ovarium, and to a lower level in the skin. These results were concordant with the transcription levels that were obtained from the RNA-seq data, suggesting that the different genes in the *muc5* family have tissue-specific transcription patterns. Similar tissue distribution and transcription patterns were also observed in other species. For example in zebrafish, different *muc5ac* genes were transcribed in skin, gill, pharynx and esophagus [33]. In common carp, *muc5b* transcripts were detected in the skin [34] and in humans different Muc5ac transcripts were transcribed in the respiratory tract, stomach, cervix and eye [35]. The transcription profile of the *muc2* family were also similar to the transcription profiles found in other species. *Muc2* seems to be dominantly transcribed in the gastrointestinal tract of gilthead sea bream (*Sparus aurata*) [36], zebrafish [33], common carp (*Cyprinus carpio*) [34] as well as in humans [37].

In a practical manner, tissue specific distribution of the mucin genes may be important in order to understand host pathogen interactions in mucosal tissues. Previous studies have shown that bacteria, such as *Aeromonas salmonicida* binds mucins isolated from the intestinal tract to a greater extent than skin mucins [38]. Further, it is also shown that the glycosylation pattern of mucins is different in the skin and intestine of Atlantic salmon [39]. Thus, knowledge on the tissue distribution of different mucins could explain the observed differences between resistance and susceptibility for infectious diseases. In conclusion, genes in the Atlantic salmon *muc5* family have a wide tissue distribution, while genes in *muc2* family were mainly transcribed in the intestinal regions. These results are in concordance with the transcription profiles of *muc2* and *muc5* in other teleost and mammalian species. Our results bring us one step closer in understanding of the biological effect of the different mucin families.

Mucin transcription in gill, intestine and skin after acute handling stress

Short-term (24 h) effects on mucin transcription in large post-smolts (~300 g) after acute handling stress was examined in the main mucosal tissues; gill, mid-intestine and skin. Results showed that the stressor led to different transcription patterns for the respective mucins depending on tissue and gene (Fig 5). In the gill, stress significantly induced transcription of *muc2.1/2* (0.94 to 1.43 fold) after 3 and 24 h. Hence, while being transcribed mainly in the intestinal region (Fig 4), *muc2.1/2* seems to have a role during stress-induced responses in the gill. Transcription of *muc5ac.2/4* also increased significantly 0.8 fold, 3 h post stress, while returning to base-line levels after 24 h (Fig 5). These genes had the highest transcription levels in the gill (Figs 2B and 4), and thus seems to be important for regulating mucus production both under normal (steady-state) and perturbed conditions. In the mid-intestine, handling stress had generally little impact on gene transcription, and only *muc5b* had altered transcription levels, being significantly down-regulated -1.54 fold after 24 h. A similar response was observed in the skin, with *muc5b* being significantly -1.37 and -0.71 fold down-regulated 3 and 24 h post stress and *muc5ac.2/4* being significantly -1.74 and -1.8 fold down-regulated at 3h and 24h compared to pre-stress levels.

The reason why the mucin transcription is differentially regulated in different tissues may be due to the metabolic costs of stress [40]. Gills are the primary organ for respiration, and

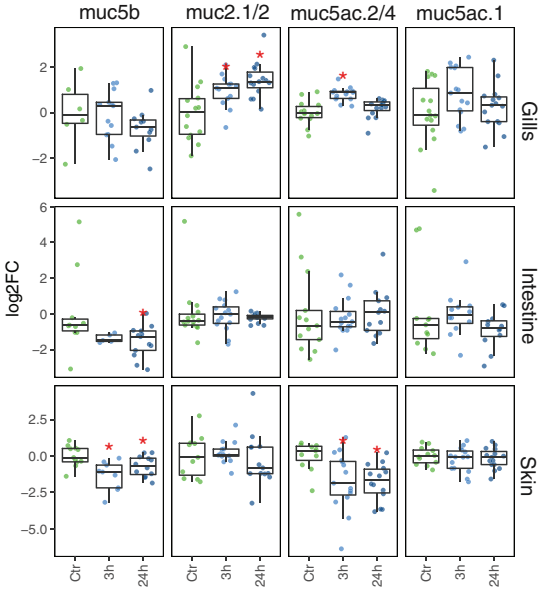

Fig 5. Changes in mucin transcription in response to an acute stressor. Differential transcription of mucin genes in gill, middle intestine and skin in response to handling stress (n = 15). On the X-axis, control (C), three hours post stress (h3) and twenty-four hours post stress (h24). The box-plot representation shows the median value of mRNA transcription (bold line), the lower and upper limits of each box representing the first and third quartiles, respectively. Whiskers represent the limits of extreme measurements. Transcription is displayed as $\log_2$ fold changes relative to the mean transcription of the mucin gene in the control group of the respective tissue. A red asterisk indicates that the marked group is significantly different compared to the control group (t.test; p.value < 0.05).

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handling stress dramatically increases oxygen consumption [41, 42]. Thus, we suggest that increased mucin transcription in the gill may be an associated effect of increased respiration. On the other hand, the observed decreased transcription in the skin and intestine could be an energy saving response. Thus, the observed differentially transcription patterns may demonstrate a differentially coping mechanism balancing the energy demand in the different tissues.

Further, we see that mucin genes belonging to the same gene family is differentially regulated in the same tissue. *Muc5b* and muc5ac.2/4 are down regulated by handling stress in the skin, whereas *muc5ac.1* is unaffected. Apart from *muc5ac.1*, of which the transcription was unaffected upon stress in all three tissues, the respective mucins could represent tissue-specific biomarkers for evaluation of effects of handling stress such as transportation and vaccination.

The practical aspects of these observations should also be taken into account, as a non-intact mucus layer is associated with increased risk of secondary infections [43, 44]. Recently high mortalities are reported as a result of mechanical de-lousing, which both stresses the fish and cause damage to the skin and the mucus layer [45]. Here we show that it takes more than 24h before the mucin transcription returns to basal levels in the skin and intestine. If the fish is exposed to rough handling procedures, such as de-lousing, the fish should be given appropriate time to recover (> 24h) in order to restore the protective mucus layers.

Mucin transcription in the skin after intensive rearing and confinement stress

Mucin transcription in the skin of post-smolts reared under intensive conditions (i.e. high biomass) followed by confinement stress was examined over a period of 14 days.Transcription of *muc5ac.2/4* increased significantly already after 24 h and kept up-regulated until the end of the experiment (Fig 6). Similar induced transcription was observed for *muc5b* and *muc5ac.1*, but at lower levels and a slower pace, being significantly upregulated first after 7 days and slightly declining thereafter (Fig 6). These findings are in line with previous results showing increased transcription of mucin-like genes in the skin with increasing fish density [46].

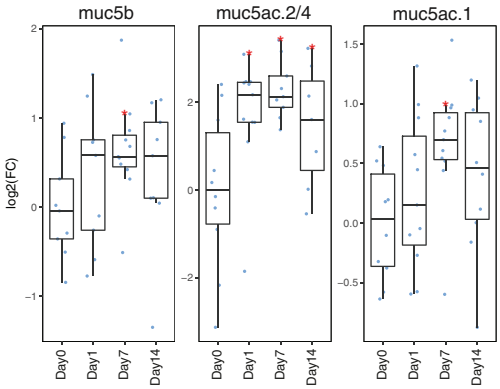

Fig 6. Mucin transcription in the skin in response to intensive rearing and confinement stress. Differential transcription of mucin genes in the skin in response to confinement stress (n = 12). Day 0 represent samples taken three days prior to introduction of confinement stress and day 1, 7 and 14 represent time after introduction to confinement stress. Statistics as in Fig 5.

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Thus, the two experiment that was conducted had different impact on mucin transcription in the skin. Netting and air exposure led to a reduction in mucin transcription, whereas confinement stress followed by high rearing intensities led to increased mucin transcription, indicating that transcription may be differentially regulated by different stressors. In the first experiment, instead of investing energy into the mucus production, which is energy demanding given the large size and heavy glycosylation of these proteins, energy could be allocated to metabolic processes such as glycogenesis which is required for this high energy demanding period. In the second experiment, the fish is exposed to a chronic stressor. In most cases, when fish are exposed to a stressor for a long period they will adapt to the condition [47]. The increased transcription of mucin genes could therefore be an adaptive response to long term stressful conditions. It is also likely that increased fish densities would lead to increased contact between fish and potential abrasion that could stimulate mucin production. High fish densities will also change the water quality parameters in the tank, which also could be a reasonable explanation for the increased mucin transcription in the skin. It is therefore unknown whether the observed increase in mucin transcription in the second experiment was due to more persistent changes in water quality parameters in the tank, skin abrasion or as a specific response to the confinement stress itself.

Future studies should focus on separating the effects of relevant environmental and biological factors on mucin production and transcriptional regulation, in order to further evaluate the application of these mucins as biomarkers for aquaculture-relevant perturbations.

Conclusion

Seven putative mucin genes were identified in the Atlantic salmon genome. Their transcription patterns show that the tissue-specific transcription pattern of Atlantic salmon *muc2* and *muc5* families are similar to those in other species. Furthermore, the results from two controlled stress experiments show that mucin transcription is regulated in response to aquaculture-relevant stressors, but the response varies depending on the type of stressor. Acute handling stress by netting led to increased mucin transcription in the gill, while it decreased in the skin and intestine. Conversely, long-term intensive rearing conditions and confinement stress led to increased mucin transcription in the skin. Our results will provide an important foundation for better understanding of mucin biology and mucosal health, and their usefulness as biomarkers for aquaculture applications.

Supporting information

S1 File. Supplementary data containing qPCR-raw data and additional primer information, as well as information about the sequences used for building the phylogenetic tree.
(XLSX)

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