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.

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 (Norwegian Ministry of Trade, Industry and Fisheries, 2011). However, since these systems carry with them high investment- and running costs, a high production intensity is required (Iversen et al., 2013; Terjesen, 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 & Fromm, 2012; Günzel & 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 & 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 & 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 & Saha, 2012a) and high concentrations of ammonia (Choudhury & 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.

Key words: closed-containment systems, skin health, fish density, specific water flow, fish welfare

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 ^{o}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 ^{o}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 & 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 $\pm$13.6, mean length (SEM) 22.2 cm $\pm$1.4) were transported from the hatchery (Flateråker) to ILAB and distributed

randomly among ten 1 m^2 square fiberglass tanks (500L) 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 09.00-10.00 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 09.00-16.00. On October 10th 2012, 2500 smolts (mean weight (SEM) = 113.6 g $\pm$ 11.8, mean length (SEM) = 22.0 cm $\pm$ 0.99) were randomly distributed among eight 1m^2 square fiberglass tanks (500 L, stocking density 75.0 kg/m^3) each with a specific 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 hours 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 x 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 1x PBST (phosphate buffered saline with 0.05% Tween-20) with 0.5% Triton x100 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 1x 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 (0.5x0.5 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 sec. at 5000 rpm with a pause of 5 sec. 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 500ng 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. Quantitative real time PCR (RT-qPCR) was conducted using 2x 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 sec, 60 °C for 15 sec, 72 °C for 15 sec (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 more than 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. 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.

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 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.

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 & 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 & 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 & 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 & 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 & 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 & 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 & 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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610

611

612 Table 1 Water quality parameters from the specific flow experiment (n=2 tanks). Average values (± SE)
613 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
sTotal ammonia nitrogen (mg/l)	0.36±0.05	0.35±0.05	0.48±0.07	0.76±0.11

614

615

616 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

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

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

Figure legends

Figure 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.

Figure 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*

Figure 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) l*ysozyme* 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 0.2-0.3 kg/l/min compared to 0.4-0.5 kg/l/min (t-tests) are indicated in the figure with p-value.

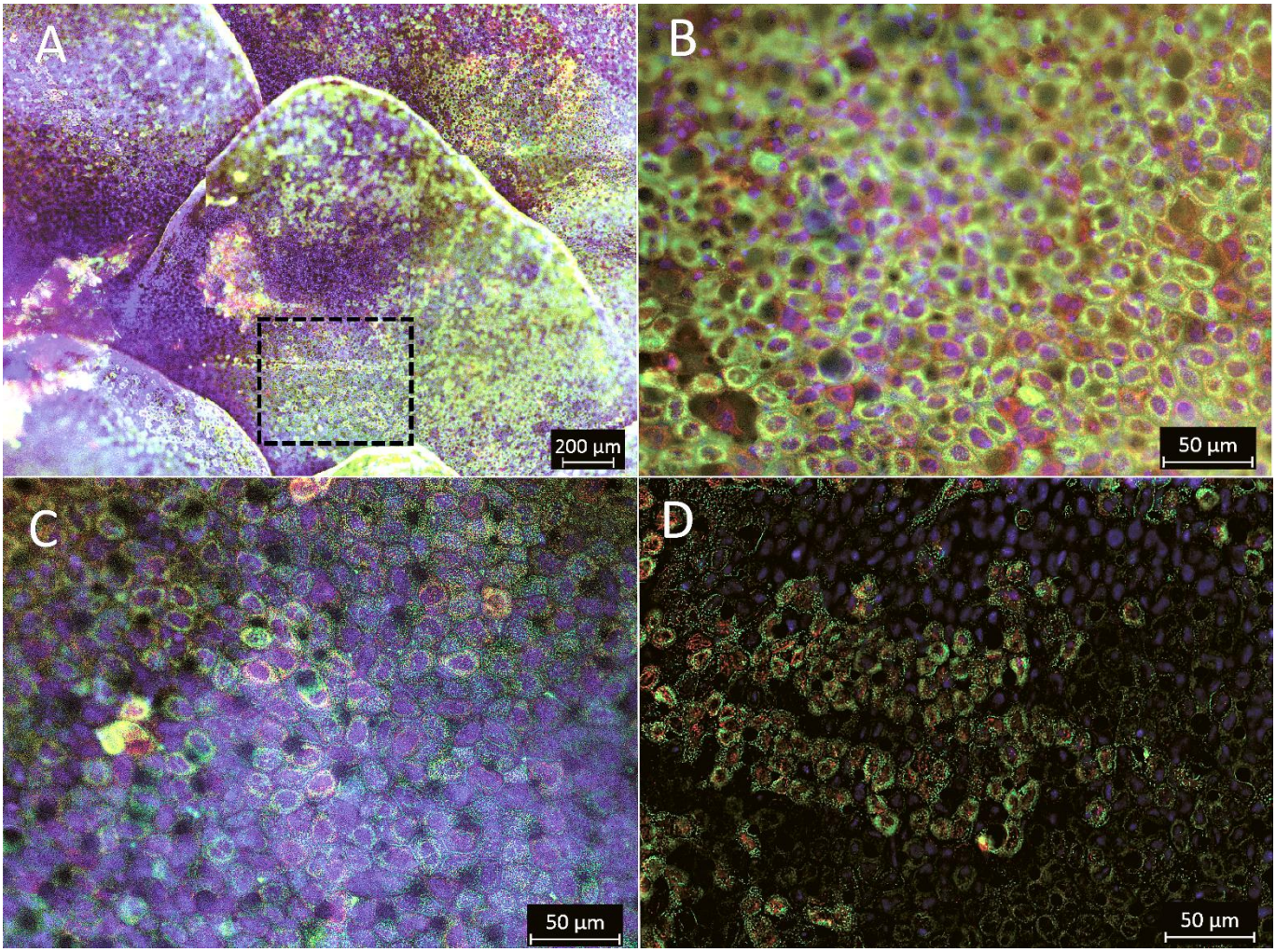
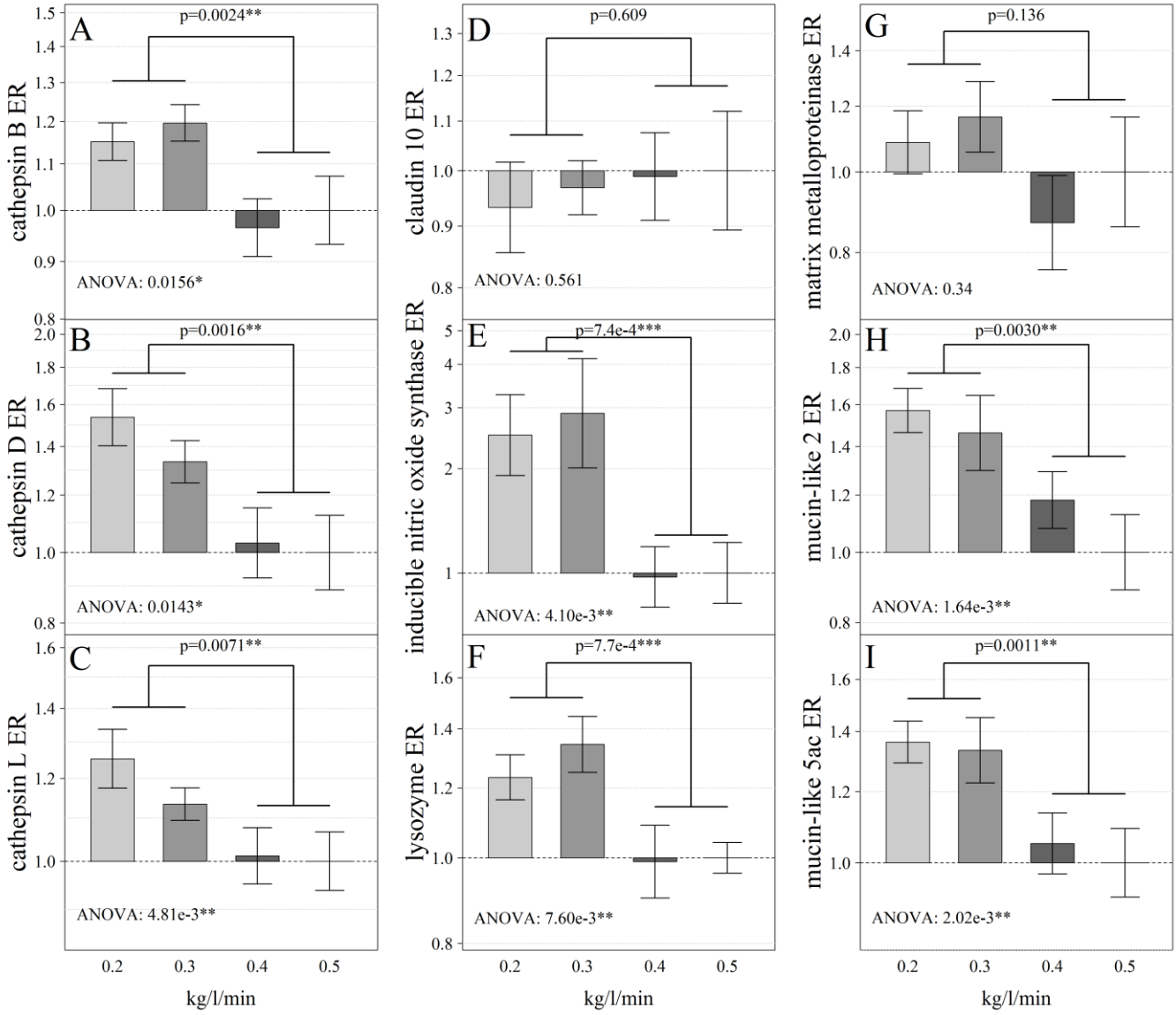


Figure 1.

659



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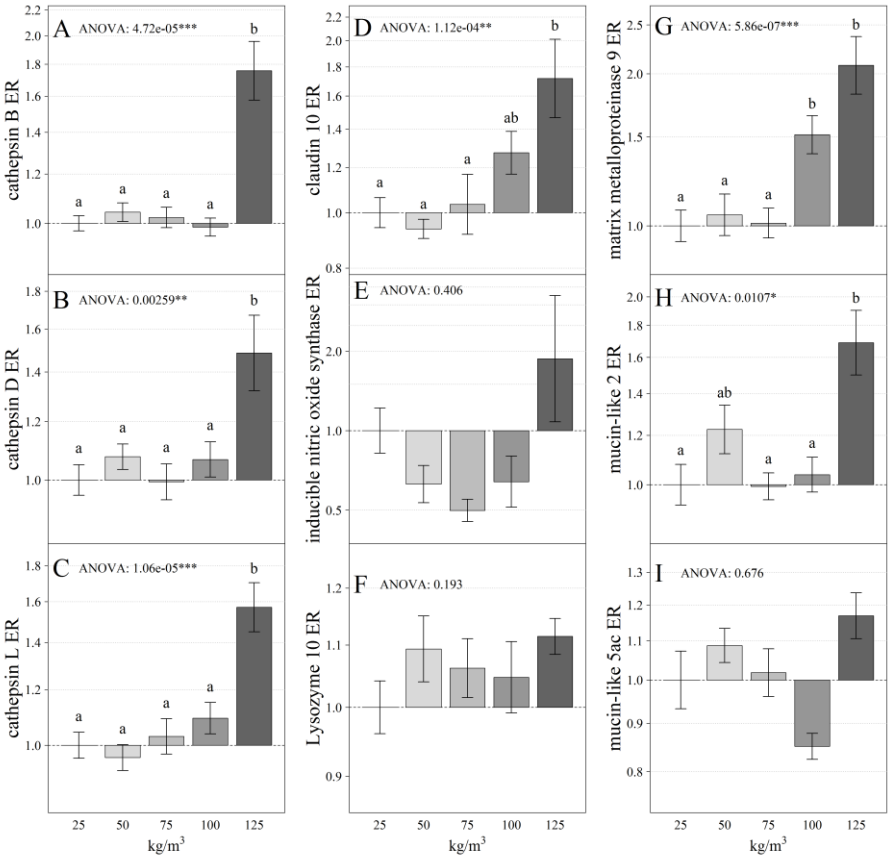
661 Figure 2


A ANOVA: 4.72e-05***
B ANOVA: 0.00259**
C ANOVA: 1.06e-05***
D ANOVA: 1.12e-04**
E ANOVA: 0.406
F ANOVA: 0.193
G ANOVA: 5.86e-07***
H ANOVA: 0.0107*
I ANOVA: 0.676
cathepsin B ER
cathepsin D ER
cathepsin L ER
claudin 10 ER
inducible nitric oxide synthase ER
Lysozyme 10 ER
matrix metalloproteinase 9 ER
mucin-like 2 ER
mucin-like 5ac ER
kg/m3

662 Figure 3

Whole-mount skin samples from fish (n=12) at each density were stained and ranked based on a standard scoring system to show the effects of fish densities on cell morphology, mucous cell density and mucus content. Mean ranks with ± standard error are shown in the table. Significant differences were marked bolt. 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
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

Effects of specific water flow on cell morphology, mucous cell density and mucus content**.** Whole-mount skin samples from fish (n=12) at each density were fluorescence stained and ranked based on a standard scoring system. Mean ranks 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
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

663