Deep neural network analysis - a paradigm shift for histological examination of health and welfare of farmed fish

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Abstract

An artificial intelligence model (AI-model) was trained for the first time to detect multi-class segmentation of skin from Atlantic salmon, using a convolutional neural network (Aiforia®). The AI-model was developed to produce reliable spatial measurements of all the successive skin layers of Atlantic salmon. The AI-model was tested on skin samples collected from eight post-smolts (produced in a research facility), with the intention of comparing skin samples from six different body sites. The results from the AI-model were highly correlated to manual measurements carried out by two experienced histologists and indicated that the abundance of epidermal and dermal skin tissues vary with body-site. The AI-model was further used to evaluate skin samples from commercially farmed Atlantic salmon. The samples were taken regularly through a production cycle (autumn 2018 to autumn 2019) and followed major operational events such as transport and de-lousing. Results from the AI-model reviled dynamic behavior of the skin, reflecting spatial changes of skin tissues related to time in the sea, life stage and operational events.

Our work illustrates how unbiased datasets from histological analysis open new possibilities for comparative studies of Atlantic salmon physiology. With time, a better understanding of tissue dynamics in relation to production and diseases may arise from automated tissue analyzes.

1. Introduction

In order to produce healthy food for a growing population, aquaculture is considered the most efficient and sustainable way of meeting the increased food demand (Food and Agriculture Organization of the United Nations, 2018). To secure growth within this sector, optimal health, and welfare of farmed animals, are crucial. However, the likelihood of disease-related outbreaks and reduced welfare increases with intensification and expansion of the production systems. Diseases are now a primary constraint to the farming of Atlantic salmon (*Salmo salar* L.) in Norway (Hjeltnes et al., 2018; Kristoffersen et al., 2018). Furthermore, fish welfare has gained increasing focus from several stakeholders, including the industry itself, national and international policy makers, non-governmental organizations, and consumers.

Skin, together with the gill and the gut, are the primary mucosal barriers in fish. High demands of barrier functions are required, as their surfaces protects the fish from the external environment (Beck and Peatman, 2015). Fish skin and the mucus layer have critical roles in protecting the animal from the surrounding environment (Esteban, 2012; Shephard, 1994; Sveen et al., 2020). The skin also carries out numerous additional roles such as sensory perception, locomotion, cellular signaling, communication and camouflaging (Elliott, 2011; Groff, 2001). External and internal factors can change both the structure and the function of successive skin layers; Both the thickness of the successive skin tissues and mucous cell number will change and adapt to the environment (Jensen et al., 2015a; Jensen et al., 2015c; Karlsen et al., 2018; Sveen et al., 2017).

Histology is the branch of biology which studies the microscopic anatomy of biological tissues. Histological analysis of Atlantic salmon skin is part of routine work for the assessment of fish welfare (Bruno et al., 2013; Roberts, 2012), as well as a current field of intense research

(Karlsen et al., 2018; Mota et al., 2019; Sveen et al., 2018; Sveen et al., 2016). Traditionally, histological evaluation of skin tissue sections is subjective, with human observations describing the skin structure in response to a given treatment or disease (Iger et al., 1988; Iger et al., 1995; Iger and Wendelaar Bonga, 1994; Kalogianni et al., 2011). The credibility of the analysis, relies on the experience of the histologists, type of scoring system, quality of samples and the outcome is prone to both human bias and errors (Wolf et al., 2015; Wolf and Maack, 2017). Thus, the goal of standardized machine-based measurements is to limit human error, produce unbiased results and reproducible data (Penttinen et al., 2018). There are of course additional differences between the information gained by human observers and an artificial intelligence model (AI-model). Whereas the AI-neural network contributes with standard measurements which allows for systematic comparison between groups, human evaluation can provide insight into new features such as a-typical morphology and disease patterns. Therefore, when evaluating an AI-model, whether and how the AI-model fits its purpose must be considered (Albert et al., 2019). In Atlantic salmon, the unicellular epidermal mucous cells can be automatically quantified by stereology (Jensen et al., 2015b; Jensen et al., 2015c; Pittman et al., 2013; Pittman et al., 2011). Unlike other imaging tools, such as Image J (Schneider et al., 2012) and CellProfiler (Dao et al., 2016) which base image segmentation on thresholding, the Aiforia® platform offers supervised machine learning based on a convolutional neural network (CNN) (Penttinen et al., 2018). Implementation of CNNs in biological image analysis have produced promising results such as accurate spatial measurements of tissues and cell types (Albert et al., 2019; Kraus et al., 2017; LeCun et al., 2015).

The present work address two sub-goals related to exploitation of a commercially available CNN toto evaluate fish health. First, we sought to develop an AI-model on the Aiforia® platform, which produces reliable spatial measurements of the successive skin layers of Atlantic salmon. Second, we verified the AI-model on two independent sample sets: Samples collected

from fish reared under controlled conditions in a research facility and samples from production fish collected from a commercial fish farm. In the first set of samples, the AI-model was tested on skin from eight post-smolts, with the intention of comparing skin samples from six different body sites. The results were highly correlated to manual measurements carried out by two experienced histologists and showed differential abundance of epidermal and dermal skin tissues from the different body-sites. On the second sample set, the AI-model was further tested on only one of the regions identified in the first round. Skin samples from commercially produced Atlantic salmon showed a dynamic behavior of the skin, reflecting spatial and temporal changes of skin tissues related to life stage and operational events. Overall, this is the first report using an AI-model to analyze histological samples from fish. The procedure could lead to a paradigm shift in how we assess fish health through histology, by opening for a numerous of possibilities linking analytical tools and diagnostics in aquaculture.

2. Materials and methods

2.1 Tissue samples

Tissue samples from Atlantic salmon were collected from three different geographical locations (Table 1). Fish from the first location were bought from the Centre for Fish Research, University of Life Sciences (NMBU), AAs, Norway. Skin samples (1.5 cm^2) from eight Atlantic salmon smolt (N = 8), approximately 500 g, were collected in six different body sites on the left side of the fish (Fig. 2 A). Four samples were taken above the lateral line, and two samples directly beneath the lateral line.

Fish skin samples from the second and third location originated from commercially produced Atlantic salmon (Table 1). Fish from the second location were produced in land-based facility recirculating aquaculture system in Troms municipality, Northern Norway. The 18th of August

the smolts were transferred to the third location, a commercial fish farm housing an R&D license owned by Aquaculture Research Station in Tromsø, Norway. The fish were kept in four sea cages. Skin samples (area 3, Figure 2 A), were taken at five time points during the production cycle in sea (Sept 2018 – Oct 2019, table 1), five fish per cage. Area 3 was chosen based on results in this trial (low variation in epidermal and dermal layer between individuals, see results, section 3.3). Prior to sampling, all fish were euthanized with a lethal dose of anesthetic (MS-222). Each month the cause of mortality was reported by the local fish health service for the locality.

Table 1. Origin of skin samples and relevant production data. The skin samples originated from three geographical locations, 1st location (Aas, south east Norway), 2nd and 3rd location (Troms, Northern Norway). The 1st and 2nd location were land based (LB) facilities with fresh (F) water. Fish from the 2nd location was transferred the 18th of August to the 3rd location for on growth in open net-pens (ONP). The indicated sea water temperature was measured on five meters depth at the day of sampling. Number of samples analyzed by the AI-model as indicated. Lice pressure and treatments as reported by the local fish health service.

Year	**Sampling**	**Location**	**Weight** (g ± stdv)	**Water**	**Production**	**Temp (°C)**	**Samples**	**Main operational events**
2018	Oct	1	500	F	LB	-	48	
2018	July	2	107.9 ± 28.8	F	LB	13	15	
2018	Sept	3	328.6 ± 91.0	S	ONP	10.8	14	SLICE® treatment, > *Caligus elongatus*/fish as counted in two cages
2019	March	3	1287.0 ± 343.3	S	ONP	3.5	16	
2019	June	3	1243.0 ±475.6	S	ONP	8.6	8 pre-transport 10 post transport	Fish transportation by well boat from small to large net-pens.
2019	Sept	3	2993.5 ± 456.2	S	ONP	11.5	9 pre-hydrolicer 9 post-hydrolicer	Second treatment with hydrolicer, > 0.5 *Lepeophtheirus salmonis*/fish
2019	Oct	3	4185.3 ± 774.9	S	ONP	9.4	6 silver coat 13 mature males	

2.2 Sample preparation

Tissue samples were stored in 10% formalin pots (CellStore™ 20 ml Pots, CellPath). Embedding, sectioning, and staining of the tissue samples were done at two locations, the Norwegian Veterinary Institute in Harstad, Norway and at Nofima, Aas, Norway. In brief, the tissue sections were hydrated in water and stained with 1% Alican blue (Alfa Aesar) in 3% acetic acid for 15 min, transferred to 1% periodic acid (VWR) for 10 min, followed by Schiffs (Sigma-Aldrich®) reagent for 15 min, and at last 30 sec in heamatoxylin (VWR) before dehydration and mounting. AB/PAS staining stain mucous cells dark blue, purple or pink based on the acidity of the mucins, while the successive skin tissues obtain different shades of pink and blue. AB/PAS stained tissue sections of Atlantic salmon skin were scanned with Aperio slide scanner (Leica) and uploaded to the Aiforia® platform.

After sample preparation, digitalized tissue sections, were uploaded to the Aiforia® platform. The regions of interest (ROI) were manually drawn onto each tissue section, avoiding areas with artefacts such as cracks and discoloration. The number of processed samples, and actual samples included in the AI-analyzes, is included in table 2. Some samples were excluded from analysis only due the presence of artefacts (poor fixation, cracks, discoloration, sampling artefacts), which made them unsuitable for AI-analysis.

2.3 Training the AI-model on the Aifoira® platform

The AI-model was trained on scanned tissue sections as described by Penttinen et. al. 2018. In brief, 122 digitalized skin sections were uploaded in the Aiforia® cloud-based management platform (Fimmic Oy, Helsinki Finland) (Fig. 1). The main segment layer identified the epidermal and the dermal layer. This layer was further subdivided to identify tissue and cell

types within the epidermal (mucous cells) and dermal layer (scales, loose connective tissue, dense connective tissue, dark pigment). All segment layers were set to very complex, with similar augmentation (Scale (-1, 1.01). Aspect ratio (1), Maximum shear (1), Luminance (-1,1), Maximum white balance 1, Noise 0). The context size assigned to each layer was slightly larger than the tissue of interest. Annotation and training regions were manually drawn to differentiate between the different cell types and tissues. Similarly, an object layer (fixed object size 18 µm) was created to differentiate between blue and purple mucous cells within the epidermal layer. After each training, the ability of the program to recognize the skin tissues were manually assessed in the validation tool provided on the Aiforia® platform. Further analysis of tissue sections was provided by the Aiforia® platform and color overlay inspected. The color overlay represents tissue and cell detection of the neural network. After repeatedly trainings (36 trainings, 2164 regions), a final training of 4000 iterations, the AI-model was deployed on the skin samples of interest.

2.4 Manual verification of the AI-model

Digitalized tissue sections from the first geographical location (n = 48) was used for the verification of the AI-model. The neural network was run on two manually drawn regions of interests (ROI), one large ROI (L_ROI) covering the entire length of the tissue section (approximately 1.5 cm), and one small ROI (S_ROI) approximately 2 – 4 mm in length. The neural network was run in both regions and the data was exported on to a local hard drive. Manual assessment measurements of the skin samples were done independently by two researchers. The manual measurements were done in Image J in an area corresponding to the S_ROI and performed on 17 samples. On each sample, six measurements were carried out to measure the thickness of the epidermis and the dense connective tissue, and the total number of blue and purple mucous cells in each S_ROI were counted.

2.5 Data analysis and statistics

To process the data, generated by the Aiforia® platform, a R-script (https://www.r-project.org/, version 3.5.2) was developed that read-in the results for each sample, filtered for quality, normalized to the sample size and combined the data for further processing. During development of the script, cutoffs and rules for each skin component for filtering were defined based on manual evaluations. For dermis and dense connective tissue only one large area for each sample was expected. In case more than one area was found, only areas, which had at least 10% of the size of the largest fragment were kept and combined to single entry. The remaining components were filtered according to calculated class confidence. The required cutoff values for the different components were the following: epidermis 0.78, mucous cells 0.8, loose connective tissue 0.7, scales 0.8, scales connective tissue 0.7 and dark pigment 0.7. Blue and purple cells were not filtered in this step. The length of each sample was calculated based on the bounding box of the dermis areas. In case of small samples, the length of the longer edge of the rectangular bounding box was sufficient as a definition of the length. Longer skin samples on the other hand were curved and the length was approximated by the long and short edges of the box, by calculating the hypotenuse length of the triangle of the shorter edge and half of the longer edge, times two (formula: sample.length = 2 * sqrt((long.edge/2)^2 + (short.edge)^2)). The calculated lengths were used in the further analyses to normalize to area in μm^2 per millimeter of skin. A report was generated for each sample and a file containing warnings in case unexpected results were found, which simplified manual revision of results. The filtered and combined results were further analyzed in R. One-way ANOVAs, Tukey *post-hoc* tests and Pearson correlations were calculated by functions of the provided *stats* package (aov(), TukeyHSD() and cor.test()). Results were plotted with a combination of custom functions and the *beeswarm* package.

3. Results and discussion

3.1 Development of the Aiforia® skin AI-model

After repeated trainings (500 iterations and 1002 manual annotated regions) on samples from our skin database, the AI-model managed to separate well between the two major skin compartments (epidermis and dermis), the mucous cell area, blue and pink mucous cells, scales and dark pigment (Fig. 3 and video 1). These anatomic structures are distinct in terms of shape and color. The deep neural network had some difficulty in distinguishing between the two major dermal compartments, the stratum compactum and the stratum laxum. The stratum compactum is mainly composed of irregular dense connective tissue with closely packed collagen fibers in alternating directions (Summers and Long Jr, 2005; Wainwright et al., 1978). The stratum laxum is made by loose connective tissue characterized by multidirectional weave of extracellular fibers (collagen, reticular, elastin), supporting blood vessels, nerves and pigment cells (Fig. 3) (Elliott, 2011). In some cases, separation of the two main connective tissues compartments can be challenging even for trained human observers (Fig. 3). The orientation of the tissue, and the presence of artefacts will to a large degree influence the visual appearance of the connective tissue. Defining the ground truth for the border between these two tissues was therefore an important task in developing the skin AI-model. By increasing the number of iterations (4000 iterations, 2164 manually annotated regions) the classification of the dense and loose connective tissue improved (Fig. 3). Thus, the CNN on the Aiforia® platform is able to classify and distinguish tissues with similar phenotypes, however such classifications require more training compared to tissues and cell types with distinct features.

3.2 Verification of the AI-model

The skin AI-model was validated against observations done by two experienced histologists, measuring the thickness of the epidermis and the dense connective tissue, and counting mucous cells. These parameters are frequently used for the validation of Atlantic salmon skin (Karlsen et al., 2018; Mota et al., 2019). The Pearson correlation coefficient (R) between the two human observers were high for all measurements, epidermal thickness ($R = 0.983$ $p < 0.001$), total mucous cell number ($R = 0.995$, $p < 0.001$), and dense connective tissues ($R = 0.984$, $p < 0.001$). The correlation between the AI-model and the human observers were also high for epidermal thickness ($R \geq 0.95$, $p < 0.001$), total mucous cell number ($R \geq 0.99+$, $p < 0.001$), and dense connective tissue thickness ($R \geq 0.89$, $p < 0.001$). The largest variations in the AI-measurements to human observers were found for the dense connective tissue in sample 5.5 and 8.6 (Fig. 4, C). This was mainly due to parts of the loose connective tissue being classified as dense connective tissue by the AI-model.

We further investigated the correlation between the small and the large region of interest (S_ROI and L_ROI) for the multiple skin tissues. Apart from the loose and dense connective tissue, where small discolorations or orientation of fibers can influence the AI-classifications, as afore mentioned, the correlation coefficient was strong with most values being close to 0.8 (Table 2). This indicates that a small area of tissue is indeed representative for a larger area, which is reassuring since manual skin measurements normally are performed in multiple smaller counting frames, as published elsewhere (Karlsen et al., 2018; Mota et al., 2019). The advantage of running the AI-model on a larger area is that the effect of small errors inside a tissue section will have a minor effect on the outcome.

Table 2 *Correlation between the large and the small ROI for the area of skin tissues, and number of mucous cells. The numbers were normalized to mm of skin. The Pearson correlation coefficient (R), coefficient of determination (R^2) and p-value as indicated.*

	R	R^2	p-value
Epidermis	0.795	0.632	<0.001
Mucous cell	0.819	0.670	<0.001
Dermis	0.780	0.609	<0.001
Dense connective tissue	0.647	0.418	<0.001
Loose connective tissue	0.502	0.252	<0.001
Scales	0.840	0.706	<0.001
Scales connective tissue	0.867	0.751	<0.001
Dark pigment	0.872	0.760	<0.001
N blue mucous cells	0.781	0.610	<0.001
N purple mucous cells	0.759	0.576	<0.001

3.3 The skin tissues vary with body-site

For in depth characterization of the skin, the developed AI-model was deployed on skin samples from six different body positions. The area of epidermis, mucous cells sand scales followed the same trend, decreasing in anterior-posterior direction (Fig. 2). Literature is scarce on how the epidermal layer varies across body sites. In gilthead seabream (*Sparus aurata*), the epidermis in ventral position was thicker compared to the epidermis in dorsal position (Cordero et al., 2017), however we did not find this in Atlantic salmon. The number of purple mucous cells per mm of skin was highest in the anterior region (position 1) (Fig. 2 E), while the number of blue mucous cells per epidermal area was influenced by position (ANOVA $p = 0.033$), but no significant difference between positions was detected. In concordance to our findings, brown trout (*Salmo trutta* L.) and Arctic char (*Salvelinus alpinus*) had the highest concentrations of mucous cells on the anterior regions of the body, with low mucous cell number on the fins (Pickering 1974). As the animal moves forward in the water a laminar flow of mucus from front to back is anticipated (Pickering 1974), reducing the drag and friction of the water during swimming (Rosen and Cornford, 1971; Shephard, 1994; Wainwright and Lauder, 2017).

The dermal area decreased in the ventral-middle segment of the body (sample position 2 and 3, 5 and 6) (Fig. 2 A). Conversely, the area of dense connective tissue was highest in the dorsoposterior position (sample position 4), (Fig. 2 D). In fish, the dermis may act as an external tendon working in unison with the mechanical movement of the muscle tissue (Hebrank, 1980; Summers and Long Jr, 2005; Szewciw and Barthelat, 2017; Wainwright et al., 1978). Congruent to our findings, shark skin is more rigid in the posterior thrust producing regions compared to more anterior regions of the body (Naresh et al., 1997), with similar observations in striped bass (*Morone saxatilis*) (Szewciw and Barthelat, 2017). Hence greater area of dense connective tissue in the posterior part of Atlantic salmon skin may be an adaption to locomotion and effective swimming.

The frequency of dark pigment in the dermal compartment was highest above the lateral line, and nearly absent in the ventral part. The normal coloration of post-smolts are dark coloration on the dorsal side and lighter coloration on the ventral part of the body (Fig. 2 A), thus the distribution of dark pigment in the skin was as anticipated. The loose connective tissue was the only tissue that did not vary with sample position. This tissue is poorly investigated in fish, and we did not find any corresponding literature from which to make comparisons.

3.4 Changes in the skin tissues during a commercial production

To further test the relevance of the AI-model, we collected samples from commercially produced Atlantic salmon (four net-pens) in Troms municipality, Norway. During the production cycle the fish were sampled prior to sea-water transfer, and at five time points during 16 months of the production time in sea (Fig. 5 and Table 1). During the production cycle the mortality rates were generally low, < 3% for the four net-pens that were followed, compared to 18.9% for Troms municipality in 2019 (Sommerset et al., 2019). The observed mortalities were

highest the first weeks after sea water transfer, and towards the end of the production cycle when the fish were frequently exposed to mechanical de-lousing events (Fig. 5). Such a mortality pattern is often observed in the commercial Atlantic salmon production in Norway, where smolt quality is relevant for survival during the first period in sea. Further, frequent mechanical delousing events may result in elevated mortality rates in the later phases of the production cycle (Hjeltnes et al., 2018; Sommerset et al., 2019). In general, the health of the production fish were characterized as good between September to May by the local fish health service. From June to November the general fish health was also classified as good, however there was an increase in mortality after fish transportation to new net-pens in June, and after mechanical delousing with Hydrolicer in August (week 32 – 33), with repeated procedure in September (week 37 and 38), and October (week 44 – 45). Even after repeated mechanical delousing events, the lice numbers were still high and in week 46 the fish were bath treated with AlphaMax®. In November 2019 there was again a slight increase in mortalities, most likely related to the previous mechanical de-lousing events and skin ulcers (Fig. 5).

Nearly every event, being sea-water transfer, growth, handling, and sexual maturation resulted in changes in one or more of the successive skin tissues. The transportation event had the overall largest negative impact on the skin morphology, whereas sexual maturation led to the greatest structural changes. These findings are presented and discussed in a broader context below.

3.5 General trends in skin development

The epidermal area was stable during the production cycle, and only two major events, transportation, and sexual maturation, resulted in significant changes (Fig 6). The ratio of mucous cells to epidermal area showed larger variation during the production cycle than the

epidermal area alone. The ratio gradually decreased from Sep 2018 to June 2019, followed by an increase in mucous cell ratio from June 2019 to Oct 2019. Concurrently the water temperature gradually dropped from autumn 2018 (Sept. ~13 ^{o}C) to early spring 2019 (March ~3.5 ^{o}C), and increasing in the summer and autumn months (June ~8.6 ^{o}C, Sept. ~11.5 ^{o}C, Oct. ~9.4 ^{o}C) (Table 1). Previous research found a decrease in mucous cell populations in winter when the water temperatures are at their lowest (Wilkins and Jancsar, 1979). In the present study, the mucous cell ratio followed the main trend in temperature, however with some exceptions. The ratio of mucous cells was higher in the cold month of March 2019 compared to June 2019. However, significant differences between the two months was only observed after the transportation event that occurred in June which resulted in the lowest recorded ratio of mucous cells (Fig. 6). Despite higher sea water temperature in Sept. 2019 compared to Oct. 2019, we observed an increase in mucous cell ratio in Oct. 2019. This increase in mucous cell ratio was mainly associated with sexual maturation (Fig. 6). Based on these findings it seems that both temperature, handling operations and sexual maturation can influence the ratio of epidermal mucous cells.

Further a separation was made between mucous cells that stained blue or purple. Mucous cells that stain purple will have a higher pH, compared to the more acidic mucins which stain blue with AB/PAS (Jin et al., 2015). As expected, the number of blue mucous cells dominated at all time points and followed the general distribution of mucous cell area to epidermal area (Fig. 6 J). The number of purple mucous cells was highest in September 2018 (Fig 6 J). At the same time the local fish health service reported high numbers of the ectoparasite *Caligus elongatus* (> 5 parasites per fish as counted in two cages). In comparison, the numbers of the closely related ectoparasite *Lepeophtheirus salmonis* is strongly regulated, with 0.5 mature female lice per fish being the upper limit before treatment is prohibited (Norwegian Food Safety Authority, 2018). Thus, despite the repeated delousing events towards the end of the production cycle, the

number of *L. salmonis* were at all time points much lower than the observed number of *C. elongatus* in September 2018. We therefore speculate if the high numbers of *C. elongatus* could be the reason for the change in mucous cell color. The closely related ectoparasite (*L. salmonis*), is known to change the mucus protein composition (Easy and Ross, 2009). Earlier we also observed a tendency towards a higher ratio of purple mucous cells in wounded Atlantic salmon (Sveen et al., 2019). Thus, higher ratio of purple mucous cells could be an indication of a stress reaction in the skin. With automatic cell counting, it will be possible to identify any such relationship in future controlled tank experiments.

The correlation between mucous cell area and epidermal area was strong and positive for most time points (Table 3). Interestingly, the lowest correlation was observed in September 2018, representing the first phase in sea, and as mentioned before, the fish had high numbers of the ectoparasite *C. elongatus* at this time-point. The correlation between mucous cell area and epidermal area also dropped from 0.9 pre-transport, to 0.3 post-transport. Pittman and colleagues (2012) have previously concluded that mucous cell density as a ratio of mucous cell area to epithelium is a relatively robust measure that can be used to compare body areas as well as the effects of treatments. Our results support this statement, and mucous cell density could be useful in interpreting the health of the epithelial tissue.

Table 3. *Correlation between mucous area and epidermis area. The Pearson correlation coefficient (R), coefficient of determination (R^2) and p-value as indicated. Before (^{b*}) and after (^{a*}) handling operation, sexual mature male (M), silver color (S), p > 0.05 in bold text.*

Month	R	R^2	p-value
July 2018	0,796	0,633	<0.001
Sep 2018	**0,448**	**0,201**	**0,108**
Mar 2019	0,726	0,528	0,002
Jun 2019 b*	0,734	0,539	0,038

Jun 2019 a*	**0,554**	**0,307**	**0,097**
Sep 2019 b*	0,909	0,826	<0.001
Sep 2019 a*	0,911	0,831	<0.001
Oct 2019 S	0,984	0,968	<0.001
Oct 2019 M	0,936	0,876	<0.001

The area of the dermal compartment gradually increased during seawater phase, with an almost linear growth of the dense connective tissue (Fig. 6 K). Previously Wilkins and Jancsar (Wilkins and Jancsar, 1979) found a correlation between skin thickness and body length in Atlantic salmon through the parr, smolt and post-smolt period, suggesting that skin thickening is a normal feature for growth in length. In zebrafish, the diameter of the collagen fibrils in the stratum compactum gradually increase with time (Le Guellec et al., 2004), thus the observed expansion of the dense connective tissue could be driven by similar mechanism in Atlantic salmon.

An increase in skin pigmentation was observed with sea water transfer, and the skin pigmentation declined towards the end of the production cycle (Fig. 6 K). This observation is likely due the light conditions (Sugimoto, 2002), where transition from indoor tanks with artificial light to deep sea water net-pens resulted in large changes.

3.5 Effect of handling procedures

The transport event from small to larger net-pens in June 2019 resulted in loss of epithelial tissue and a decrease in scale area (Fig. 6 and Fig. 7) and inflammation in the subcutaneous red muscle tissue (Fig. 7C and D). Welfare scoring of 20 fish pre (n = 10, two net-pens) and post crowding (n = 20, one net-pen), indicate higher frequencies of hemorrhaging, scale loss, cataracts and focal bleeding after the transportation event (supplementary file 1) (Fig. 7). These physical injuries were likely a sum of crowding and contact with abrasives like barnacles that

were observed attached to the net of the pen and pumping in and out of new cages (Fig. 6 and Fig. 7). Given that the welfare score was only carried out on 20 fish from one net-pen post-transportation, we cannot extrapolate the extent to which these observations are applicable to all the fish under study. In the days and weeks after the transportation event, only a small increase in mortality was observed at the location (Fig. 5). This could mean that the damage caused during the operational events were not sufficient to influence mortality.

Despite being rather rough handled during the transport event in June 2019, the skin had recovered in September 2019. The fish were crowded and mechanically treated with Hydrolicer in August, and then again in September. The mechanical treatment in September did not result in any major reduction in the epidermal area, nor infiltration of inflammatory cells (Fig. 6 and Fig. 7). However, human observation of tissue sections did note areas with epidermal abrasion of the superficial keratocytes (Fig. 6 and 7). Further, photographs of the fish showed larger areas with scale loss after the treatment (Fig. 7 F). The areas of the fish body with scale loss varied between the individual fish, but was typical for the belly, and in the anterior and dorsal regions of the fish (Fig. 7 F). The collected skin samples from the production fish were taken from the middle part of the body, position three (Fig. 2 A). Thus, the area that were used for histological analysis, may not have been optimal for detection of all skin damages or recovery after mechanical treatment(s). In future studies, it will be relevant to expand skin sampling positions when investigating skin changes prior, during and after mechanical treatments. However, the processing costs for histology samples are still considerable, and the number and scale of skin biopsies will to a large degree be dependent on the budget and goals of the study.

The de-lousing events were related to increased mortalities (Fig. 5). Mortalities post de-lousing treatments is likely due to a combination of stress and bodily damage. Fish health personnel at

the location also reported low detected levels of cardiomyopathy syndrome (CMS) in September 2019. CMS is one of the infectious diseases which is rising in Norway (Sommerset et al., 2019). The disease makes the wall of the fish hearts fragile, and repeated mechanical treatments in combination with CMS frequently results in increased mortalities (Sommerset et al., 2019). Mechanical de-lousing procedures are on a general basis associated with increased mortalities and bodily damage, with possible loss of barrier function post treatment (Hjeltnes et al., 2018). How the three dimensions of skin damage is related to the development of severe pathologies in fish, is not well investigated. Preliminary data presented at the mucosal conference in Oslo, Norway (2019) (Sundh, 2019) indicated that an intact epidermal layer is necessary for the osmotic barrier function. This implies that even superficial skin damage, such as scale loss, will result in a "leaky" skin (impairing the osmotic balance). Further, it is established that even small damages to the skin, such as removal of the mucus layer, can increase the risk of secondary infections (Raj et al., 2011; Svendsen and Bøgwald, 1997). Reducing skin damage during mechanical operations may therefore be one way to secure the health and welfare of the fish.

3.6 Sexual maturation

At the last sampling, a high proportion of the sampled fish were sexually mature males. Typical secondary sexual characteristics of the males include prolonged jaws with a developed kype (Fjelldal et al., 2018), and loss of the silver color (Fig. 8). Our results suggest that the thickening of the skin in sexual mature males is driven by expansion of the loose connective tissue (Fig. 6 and 8). It has also long been known that the thickness of the connective tissue of fish skin in salmonid species is greater in sexual mature than in immature individuals, being thickest in males (Pickering, 1977; Robertson and Wexler, 1960; Stoklosowa, 1970). However, a novelty of this study is that this thickening is driven by an expansion of the loose connective tissue.

Loose connective tissue is the most diverse tissue found in the skin, with nerve cells, pigment cells, chromatophores, immune cells, fibroblasts and blood vessels (Fig. 8 D) (Elliott, 2011). For a human observer, it is difficult to perform spatial measurements of this tissue, as its irregular shape fills the space between the epidermis and the dense connective tissue (Video 1 and Fig. 8). However, as fore mentioned, the AI-model can measure this tissue with higher accuracy. In sexually mature Atlantic salmon, the drastic expansion of the loose connective tissue happened simultaneously with an increase in epidermal area with higher number of mucous cells (Fig. 6 and 8). Spawning induced changes in fish skin morphology are described for many fish species as an adaptation to: mate selection, fighting, nest building, hormone and gamete secretion (Donaldson et al., 1983). A considerable thickening of the epidermis with increased mucous cells has been reported as an adaptation to spawning in Atlantic salmon (Rydevik, 1988). We hypothesize that the expansion of the well vascularized loose connective tissue is related to the increase of mucous cell numbers in the epidermal layer, as the poorly vascularized dense connective tissue did not respond to sexual maturation (Fig. 6 K and Fig. 8). The blood vessels located in the loose connective tissue are a fine system of capillaries involved in nutrient supply, gas transfer and acid-base regulation (Glover et al., 2013; Ishimatsu et al., 1992; Steffensen and Lomholt, 1992). A higher mucous cell number in the epidermis of sexual mature Atlantic salmon is likely associated with a higher production of mucus proteins, which translates to an increased demand for nutrient. The energy demand of mucus secretion in fish is to our knowledge unexplored, however in in molluscs mucus production represents as much as 70% of the consumed energy (Davies and Hawkins, 1998). Further, transcriptional analysis of the skin of sexually mature Atlantic salmon showed large changes in nutrient mobilization (Krasnov et al., 2015), further supporting the links between expansion of the loose connective tissue and nutrient supply.

4. Concluding remarks

The AI-model showed correlations with normal histological features of the skin, enabling us to follow the development of the skin of Atlantic salmon at a new and more informative level compared to traditional histological evaluations. The main advantage running an AI-model is the generation of large and reproducible data sets which can be compared with other production parameters to discover significant biological changes. However, before developing or implementing an AI-model, we recognize that some considerations should be made. As the AI-model learns from the input data, a good quality training set and well considered annotations, are crucial for success. Moreover, the AI-model is flexible and will change as more data is incorporated into the model, continuous updating and validation of the AI-model is required. Quality control of the data processing require collaboration between data analysts and histologists to ensure output data with biological significance. A main disadvantage with the model is that pathologies are not included in the training of the model and will therefore not be recognized. This may lead to loss of information or misinterpretation of results. Manual verification of random samples and knowledge of the samples being analyzed may reduce this risk. In the long run, AI-based models for evaluation of salmon health may represent a paradigm shift in how information from histological samples are used and how health of the farmed animals is evaluated.

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Ethical statement

The animals used in this study were obtained from the Centre for Fish Research, University of Life Sciences (NMBU, AAs, Norway) and from a commercial fish farm housing an R&D license owned by Aquaculture Research Station in Tromsø, Norway, and approved by the Norwegian Animal Research Authority (NARA) for the production of aquatic animals. The fish were euthanized on site, and all samples were taken after the fish were euthanized. Thus, no approval was needed from the Norwegian Animal Research Authority (NARA). Euthanization and sampling of fish were performed in accordance with the Norwegian Animal Welfare act.

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Author contributions

E.Y., and L.S. conceived the idea for the research. L.H and E.Y. were involved in management and coordination responsibility for the research activity. G.T and L.S performed, processed, and analyzed the data/visualization. L.S. wrote the initial draft, all authors contributed to the manuscript and reviewed the final version.

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Captions

Figure 1: Workflow and tissue structure identification. A. Workflow to create the skin AI-model. **B.** Tissue sections were manually annotated with annotation regions (blue and green area) and training regions (black line). **C.** The skin AI-model was deployed on two regions of interests (ROI), large (L_ROI) and small (S_ROI). **D.** Details of tissue detection, epidermis (epi, light blue), dermis (der, orange). **E.** Further detection of skin tissues included scales (SC, pink), dense connective tissue (dct, blue) and loose connective tissue (lct, yellow). **F.** Object detection of mucous cells marked by red circles. **G.** Segment detection of mucous cell area marked with pink. The colors are presented as they appear after image analysis on the Aiforia® platform.

Figure 2: Skin samples from six different positions. **A.** Picture of fish after sampling of tissue from six different body sites. **B.** Color overlay of the epidermis (green color) with mucous cells (red circles), and dermis (red color), skin from position 2 and position 4. **C.** The two main components of skin epidermis and dermis per 1000 μm^2/mm of skin. **D.** The different dermis compartments, per 1000 μm^2/mm skin **E.** Mucous area and number of mucous cells per 1000 μm^2 of epidermis, and mucous cell number per mm of skin. Trend lines and their respective formulas for sample positions one to four were added in blue. One-way ANOVA p-values are shown in the top left corners. In case significant differences ($p < 0.05$) were found, a Tukey post-hoc test was calculated and significant differences between groups were indicated by lower-case letters besides of the respective means. Groups, not sharing a letter were significantly different to each other.

Figure 3. Verification of skin samples by the neural network color overlay of the identified tissues. **A** Scanned tissue section of Atlantic salmon skin stained with AB/PAS. **B** The neural network recognizes epidermis (blue) and dermis (green) with high precision. **C** The neural network failed to recognize loose connective tissue (lct, light blue) (box 1), while some dense connective tissue (dct, green) was recognized as lct (box 2). The neural network was trained with 500 iterations and 1002 annotated regions. Scales (sc, yellow), pigment cell (pc, red) **D** Note the small improvements in tissue detection (box 1 and 2), training with 4000 iterations and 2164 annotated regions. Digital color overlay as presented on the Aiforia® platform.

Figure 4: Human observations and validation of the skin AI-model. **A.** and **B.** Comparison of manual measurements and the AI-model for the epidermis and dense connective tissue (DCT) thickness. Each black data point shows the mean value of the six measurements that were done in each ROI, with +/- standard error of the mean (SEM). Squares indicate results of Person 1 and 2 respectively. Blue circles indicate the thickness of the tissues as calculated by the AI-model. Correlation (Pearson) estimates (R) with p-values of correlation analyses between the AI results and manual measurements by the two persons are shown in the figure. **C** Numbers of blue and purple mucous cells counted manually and by the AI-model. Bars in lighter colors show manually counted numbers (shaded bars for Person 2). The dark blue bars show mucous cell numbers calculated by the AI-model. Numbers of purple cells were stacked on top of the blue bars.

Figure 5: Cause of mortality and accumulated mortality from August 2018 to November 2019 as reported monthly by the local fish health service. Due to low mortality rates in the groups reported by the local fish health service as hemorrhagic smolt syndrome (86 fish), bath treatments (253 fish) and handling (258 fish) these data were merged with other known causes,

while ulcers, snout/fin rot and winter ulcers were merged into one group named ulcers. The months when skin samples for AI-analysis were taken are indicated in the figure (grey-shade on the x-axis).

Figure 6: Skin development during commercial Atlantic salmon production and the effect of the production environment and handling operations. A – I. Histological sections of skin. **J.** Development of epidermal area, mucous area, and mucous cell number during a production cycle, from upper to lower panel. Epidermal area per mm of skin. Ratio of mucous cell area to epidermal area. Number blue and purple mucous cell per mm of skin. **K.** The development of dermis and dermis component over time, from upper to lower panel. Dense connective tissue (DCT) area per mm of skin. Loose connective tissue (LCT) area per mm of skin. Area of scales and scale connective tissue (SCT) per mm of skin, area of dark pigment (DP) per mm of skin, before (b*) and after handling operation (a*), female/silver color (F), sexual mature male (M).

Figure 7: Handling operations and micro- and macroscopic changes in the skin. A. Pumping of fish in association to with transportation to new net-pens**. B.** Bodily damage on the belly after the transportation event. **C.** and **D.** Loss of scales (orange arrow), epidermis and inflammation in red muscle tissue after the transportation event. **E.** Crowding event prior to treatment with hydrolicer. **F.** Scale loss (orange arrow) after post-hydrolicer treatment. **G.** Intact skin after hydrolicer treatment **H.** Damaged epidermal surface was after the hydrolice treatment (green arrow).

Figure 8: The effect of sexual maturation on the skin. **A.** The skin of a sexually mature male (Oct. 2019). The loose connective tissue (lct) (pink) and epidermal layer (light blue), with

plenty of mucous cells (green) is largely expanded. **B.** The skin of an Atlantic salmon with silver coat (Oct. 2019). The dense connective tissue (dct) (dark blue) is similar in size to that of sexual mature Atlantic salmon. **C.** Photos of Atlantic salmon with silver coat (upper) and a sexual mature Atlantic salmon (lower). **D.** Differences between the loose and dense connective tissue. The border between the well vascularized loose connective tissue and the poorly vascularized dense connective tissue is marked by a dotted yellow line. Areas in the loose connective tissue with more than two blood vessels are indicated by dotted circles. Nerve cells are also frequent in the loose connective tissue, indicated by a solid circle. Note that there are no blood vessels in the dense connective tissue. AB/PAS stained tissue section. Digital color overlay as given by the AI-model.

Captions video

Video 1 Detection of the successive skin tissue with the AI-model on the Aiforia platform**.**

Supplementary file 1

The FISHWELL scoring scheme for morphological operational welfare indicators (OWI's) for farmed Atlantic salmon, pre- and post-transportation