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Dietary effects on growth, skin morphology and stress marker genes of Atlantic salmon (*Salmo salar* L.) smolts

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1. INTRODUCTION

The World's population is expected to reach 9.7 billion by 2050, representing an increase of 2 billion people in the next 30 years (UN, 2019). Consequently, there is a need for increased food production in the future (Cole et al., 2018). The aquaculture industry is the fastest-growing food sector and is assumed to be a part of the solution to meet this demand for several reasons (FAO, 2020a; OECD, 2016). First of all, arable land cannot meet the increased food demand alone (Costello et al., 2020). Only 2% of what we eat derives from the sea (Institute of Marine Research, 2019), even though it covers more than 70% of the earth's surface. Moreover, fish convert feed into edible body mass more efficiently than livestock (lower FCR) (Fry et al., 2018). Therefore, aquaculture could be highly resource-efficient, rearing organisms with high protein retention and low basal metabolism (Naylor et al., 2009; Torrissen et al., 2011). Additionally, aquatic species provide healthy food that are beneficial to our health (Troell et al., 2019).

Seafood could derive from both fisheries and aquaculture. The latter recently surpassed wild fisheries in terms of volume (OurWorldInData, 2019), and the trend is expected to continue (Garlock et al., 2020). This is because wild-caught fish is a finite resource that has exceeded its volume limit (34.2% overfished marine stocks in 2017) (FAO, 2020b). However, aquaculture has several issues that need to be solved to achieve further growth (Subasinghe et al., 2009) and meet the UN's sustainable development goals (FAO, 2020b). Therefore, concerns regarding biology, technology and economy need to be solved for several species in aquaculture today. Globally, grass carp (*Ctenopharyngodon idella*) is the most cultivated finfish species. Placed as number nine as the most intensive farmed species, is the Atlantic salmon (*Salmo salar* L) (FAO, 2020b).

Atlantic salmon grows up in freshwater until reaching smoltification, a metamorphosis that prepares the fish for saltwater (Stefansson et al., 2020). According to the Fish Health Report by the Norwegian Veterinary Institute, poor smoltification is one out of the top 10 primary welfare problems in Norwegian aquaculture. This is mainly due to osmoregulatory issues, which challenge maintaining homeostasis (Norwegian Veterinary Institute, 2021). Salmon that are not smoltified at sea transfer will have poor performance or die within a short time if released too early into the sea, and the mortality at sea transfer is high (above 10% mortality the first three months after transfer in Troms) (Noble et al., 2018; Norwegian Food Safety Authority, 2014). Therefore, robust smolts with good health are essential for good performance and welfare in saltwater (Sissener et al., 2021). However, there is only a short period when the smolt is suited for saltwater (known as the smolt window) (Sigholt et al., 1998). This makes the smoltification a sensitive process that is hard to synchronise between individuals (Porter et al., 1998).

The skin needs to be intact to function optimally (Karlsen et al., 2018). However, the Norwegian Fish Health Report highlighted wounds as a major problematic issue for on-growing salmonids. The same survey reported mechanical delicing as the primary cause of scale loss (Norwegian Veterinary Institute, 2021). Such skin implications could decrease fish welfare (Noble et al., 2018). Therefore, it is essential to determine how to improve and measure skin health (Nofima, 2021).

Besides the increased mortality in Atlantic salmon production the recent years (Norwegian Veterinary Institute, 2021), feed ingredients have rapidly changed from marine to plant ingredients (Aas et al., 2019). The feed volume in freshwater is small, but it can give a high return in later production stages if the feed has a major impact during the early stages (Sissener et al., 2021). To improve welfare during the fragile saltwater transfer, it would therefore be of interest to examine whether salmon skin could be affected by diet composition in the freshwater phase.

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3. ACKNOWLEDGMENTS

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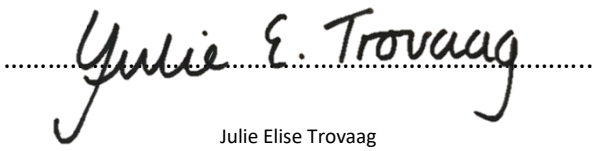
Losing two of my closest have made me realize how lucky I am to be surrounded by so many kind people. Thanks to my family and friends for always supporting me.

"*Husk da at veien blir til mens man går og at man blir rikere av hver erfaring man får»*

(Ferdinand Finne)



Julie Elise Trovaag

4. ABSTRACT

The transfer of smolts from freshwater to saltwater is a challenging period during the commercial production of Atlantic salmon. The fish undergoes profound behavioural, developmental, and physiological changes. Additionally, the first protective barrier against environmental disturbances and osmotic stress is the salmon skin. This thesis aimed to examine how diets affect Atlantic salmon smolts' skin morphology and stress marker genes. Also, the dietary effect on growth and the response to seawater transition was examined. To understand how freshwater diet could affect the smolts, the fish were fed either a marine or plant-based diet from start-feeding (30g) until seawater transfer. Thereafter, both dietary groups were mixed and fed a commercial diet for 16 days after saltwater transfer.

The data collection included examination of the skin, which involved photographing the whole fish, and skin sampling for histology analyses and gene expression. Also, the fish was weighted, and length was measured. Fish were sampled just before and 16 days after saltwater transfer. The fish fed a marine diet in freshwater had a higher body weight compared to the fish fed the plant diet in both fresh- (112 vs. 81g) and saltwater (118 vs. 89g). Furthermore, the dietary effect on skin morphology showed that the fish fed a plant-based diet in freshwater had a larger epidermis area and thicker epidermis in saltwater than those fed a marine diet in freshwater. The epidermis was limited influenced by expression levels of stress marker genes. However, the response to saltwater transition showed a reduced number of mucus cells, apically (relative to internal) mucus cells and condition factor. Scale loss was not significantly affected by saltwater transition or dietary composition.

Keywords: Aquaculture, Atlantic salmon, smoltification, fish nutrition, skin, scales, mucus, epidermis, marine ingredients, plant ingredients, raw materials, welfare.

5. OBJECTIVES

The main goal was to study the dietary effect on skin morphology (scale loss and histology) and stress marker genes of Atlantic salmon fed a diet based on marine- or plant raw materials before sea transfer (Figure 1).

Furthermore, the study had two sub-goals. These aimed to determine 1) the dietary effect on growth and 2) response to seawater transition.

The hypotheses being tested were: The diet 1) *affects* skin morphology, stress marker genes and 2) growth of Atlantic salmon smolts, and the seawater transition 3) *does* impact the response of Atlantic salmon smolts.

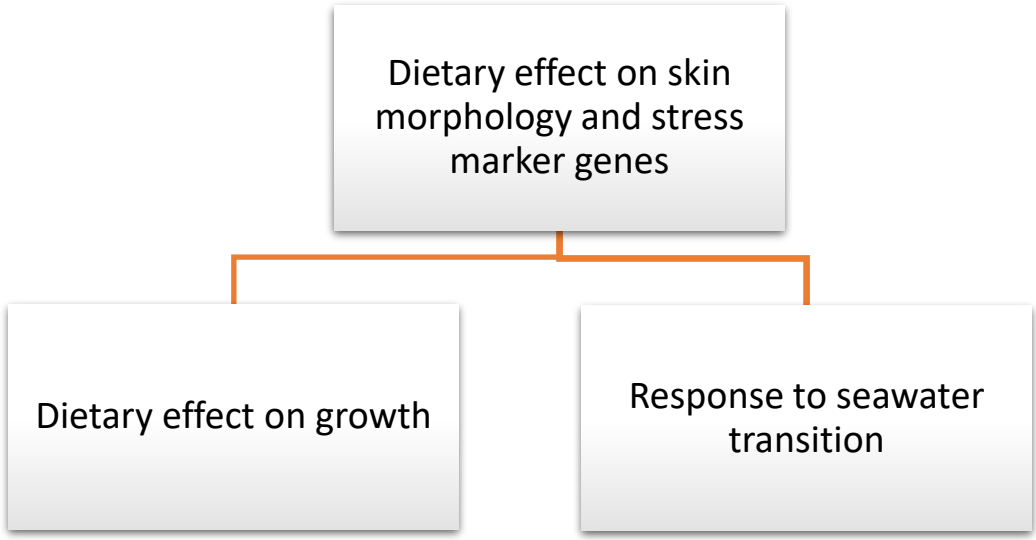


Figure 1: Hierarchy showing the main- (top) and sub-goals (bottom) in the experiment.

6. THEORETICAL BACKGROUND

6.1. SALMON FARMING

Atlantic salmon (*Salmo salar L*) is among the world's most intensive farmed aquatic species. Intensive fish farming means a production with high control through high investments, fish densities, artificial feeding and constructed facilities (FAO, Unknown year). Although the species only comprises about 4.5% of all the global finfish production (in volume) (FAO, 2020b), it receives much attention due to its high price and excellent flesh quality (sciencenorway.no, 2020). According to FAO, it was the world's second-largest aquaculture species, ranked by value in 2017 (FAO, 2017).

The top three Atlantic salmon producers worldwide are Norway (52% of volume in 2018), Chile (27%), and the United Kingdom (7%) (FAO, 2018). 94% (1.36 million tonnes in 2019) of all aquaculture species produced in Norway are Atlantic salmon (Statistics Norway, 2020). With its long coastline and cold-water temperature, Norway's natural conditions are beneficial for the mariculture of cold-water species. Hence, there are more than 1000 commercial, on-growing salmon farms (Figure 2) (BarentsWatch, 2021), and more than 100 salmon farming companies operating in Norway (Kontali Analyse AS, 2019b). Also, there are several suppliers to the industry, providing feed, genetic material, equipment and transportation along the shore (Kontali Analyse AS, 2019a).

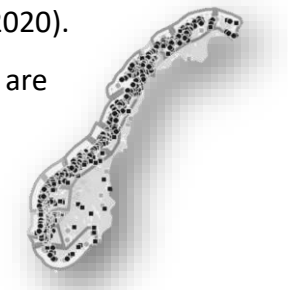


Figure 2: Map showing the distribution of Norwegian salmon farms. Photo derived from barentswatch.no/fiskehelse/

Despite the favourable natural conditions and the profitable aspect of salmon farming, the industry faces some significant issues. These issues are primarily related to salmon lice and escapees (Taranger et al., 2015). However, the Norwegian government aims to increase salmon production fivefold within 2050 (Nærings- og fiskeridepartementet, 2021). Consequently, there is much research within biology and technology going on. For instance, CtrlAQUA (Centre for Research-Based Innovation in Closed-Containment Aquaculture) is a multi-disciplinary project supported by several R&D partners, and technology, farming and pharmaceutical companies, which aim to "*develop technological and biological innovations*" for future farming methods in closed-containment aquaculture systems (CCS) (CtrlAQUA, 2021).

6.1.1. The lifecycle of domestic salmonids

Atlantic salmon are reared on land in juvenile stages (8-18 months) before transferring to seawater (Institute of Marine Research, 2021), where most fish are kept in open cages (BarentsWatch, 2021). After about 12-18 months at sea, the fish have usually reached a weight of 3-6 (5) kg and are ready for slaughter (Institute of Marine Research, 2021). Overall, its lifecycle can be divided into six stages (seven when including broodstock) (Figure 3) (Scottish Sea Farms, 2021). The time when the fish reaches each life stage is primarily dependent on day degrees/d°, meaning water temperature times days (Torstensen et al., 2004).

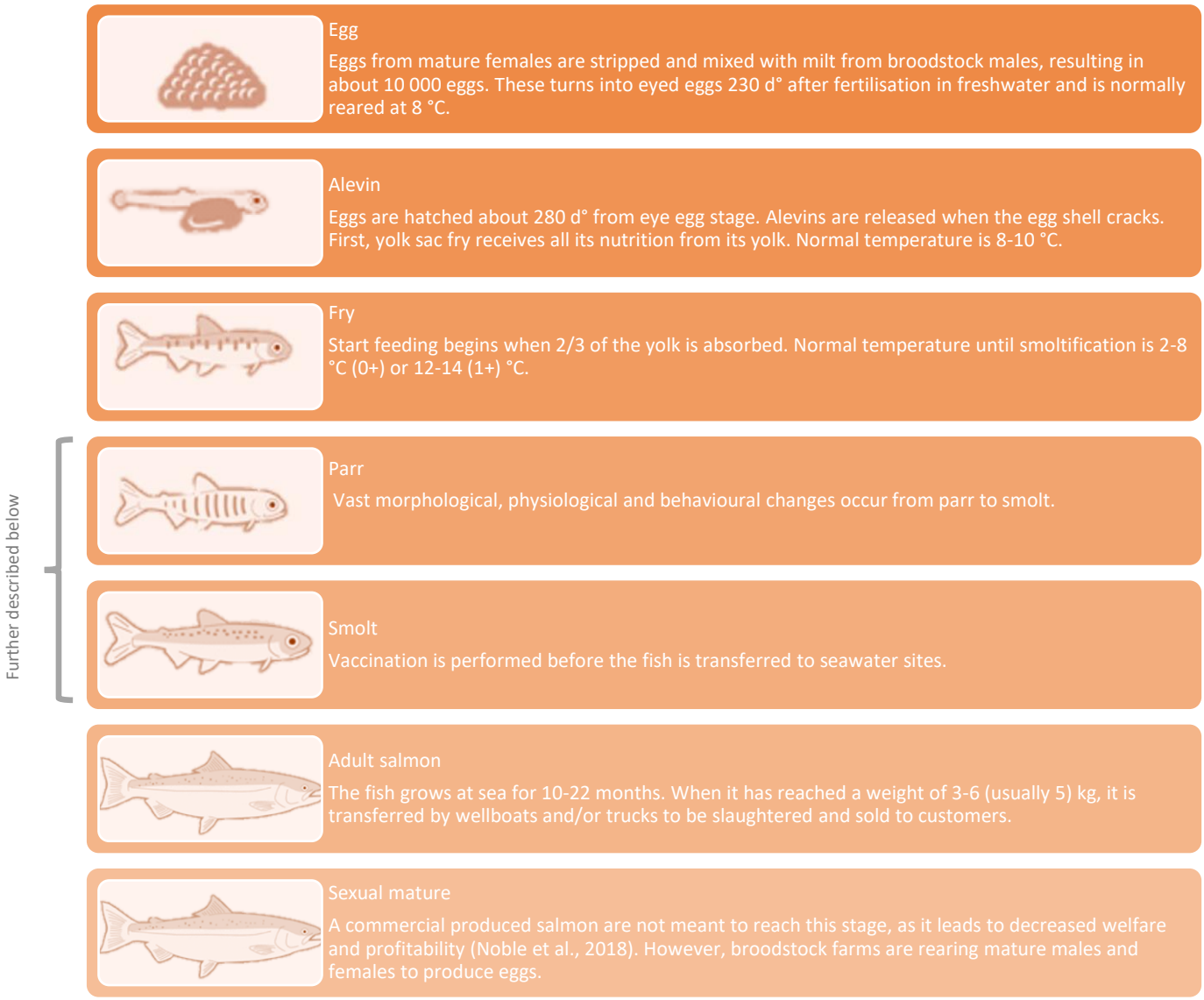


Figure 3: Lifecycle of domestic salmonids in commercial production, separated by seven life stages. Photos derived from: https://www.bestfishes.org.uk/scottish-salmon-farming/

6.2. SMOLTIFICATION

The Atlantic salmon is an anadromous species, which means that it can osmoregulate both in freshwater and saltwater (Poppe, 2019). Smoltification represents the pre-adaptation to marine environments/higher salinities, which occurs during the freshwater stage (Prunet et al., 1989). This transformation is a metamorphosis from parr to smolt that includes profound changes in behaviour, morphology and osmotic regulation. (Fleming et al., 2019) (Figure 4). These changes are further described in the following sub-chapters.

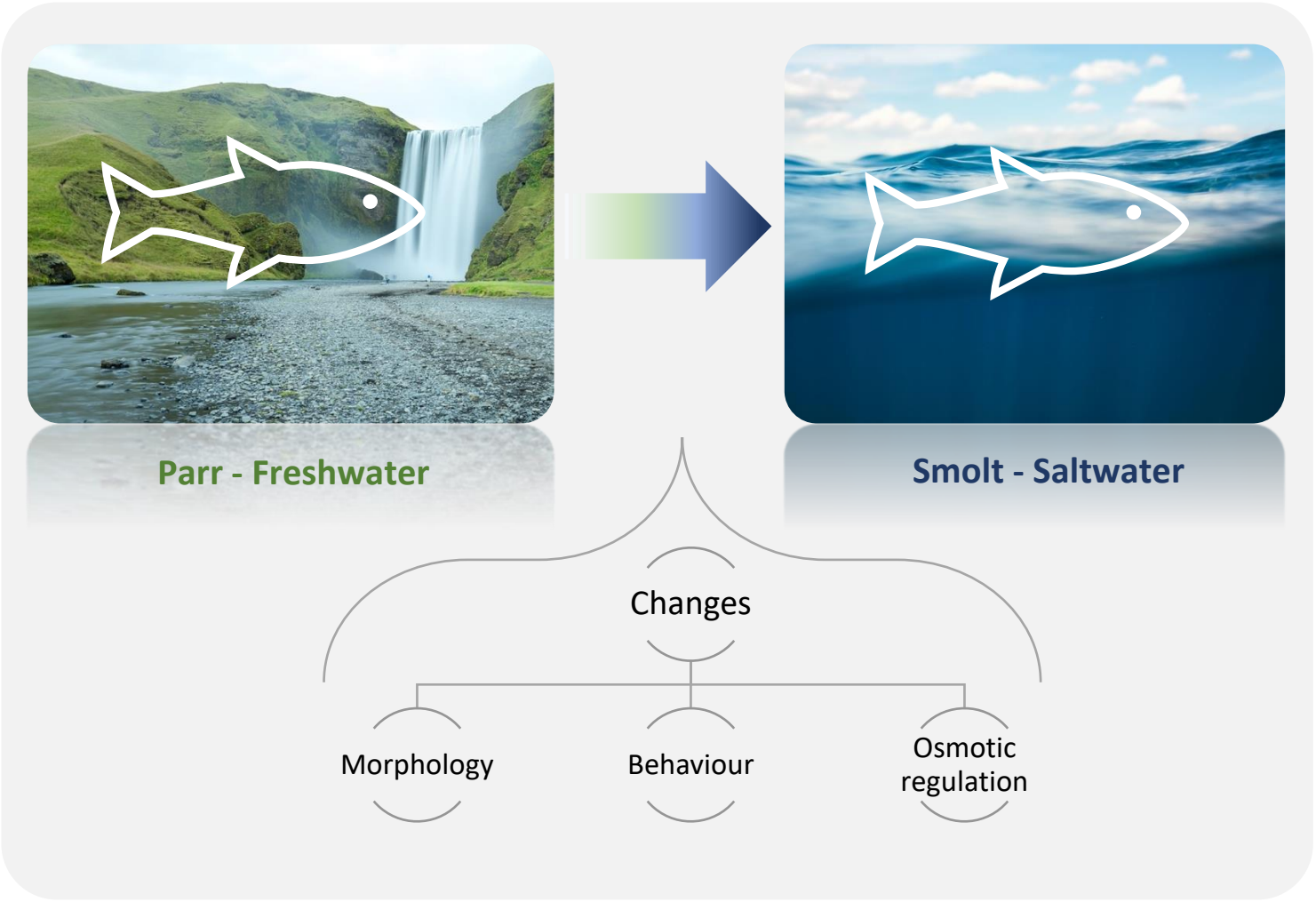


Figure 4: Illustration of smoltification – the transfer from freshwater to saltwater. Underneath the illustrations, the three most significant changes that happen during the transformation are mentioned.

6.2.1. Morphology

Parr has a green/brown colour and fins in the same tone as the rest of the body. Also, they have characteristic parr finger marks, which are vertical oval dark dots placed on the side of the fish, between their red smaller dots.

Both colour and shape change as the fish smoltifies. The parr marks and the red dots disappear, and the fish turns more silvery (Duncan & Bromage, 1998). Accordingly, the fins' edges and the back turns darker, and the become slimmer (condition factor is reduced).

6.2.2. Behaviour

Parr are quite territorial in the river. Also, it is fairly stationery and lives in a small area, calmly swimming against the stream (positive retroactive behavior) in the passive parts of the river.

Smolts in freshwater gradually increases its activity and swim to the more stream exposed parts of the river. Afterwards, it will swim more active with the stream (negative retroactive behavior).

6.2.3. Osmotic regulation

The osmolality in freshwater is around 0-50 mOsm/L. Hence, freshwater teleosts are somewhat impermeable to ions and hyperosmotic to the environment. At first, the water is kept outside the body by not drinking. Instead, water passes into the body through the gills, which are more selective than the mouth. Also, the gills regulate the active absorption of electrolytes (Na^+ and Cl^-), and the urine is dilute (Halver & Hardy, 2002).

The osmolarity in saline water is about 1000 mOsm/L. Hence, marine teleosts are hypoosmotic to the environment and excessive ions must therefore be excreted in a higher concentration than the income water. To achieve this, the fish is continuously drinking. Also, the electrolytes (Na^+, Cl^-, NH_4^+) are actively excreted through the gills, and it has a concentrated urine (Halver & Hardy, 2002).

6.2.4. SMOLTIFICATION IN COMMERCIAL PRODUCTION

Commercially produced smolts are between zero (0+) to one (1+) year when smoltification occurs (Roth et al., 2005), whereas the process in nature takes 1-8 years (Jonsson et al., 1998; Klemetsen et al., 2003). This rapid process is obtained in the industry by manipulating abiotic factors (exogenous factors) (Stefansson et al., 2018). Light and temperature manipulation are the most common, affecting the circannual cycles (Handeland & Stefansson, 2001; McCormick et al., 2007; Stefansson et al., 2007). However, "*in recent years, light control has been reduced due to increased RAS (Recirculating Aquaculture Systems) and the possibility of saline adaptation through rapid growth. Otherwise, saline feed (functional feed) was initially the first method developed to improve the problems related to saline adaptation*" (Sæther, 2020).

Osmotic regulation is the most critical part of smoltification (Stefansson et al., 2012). If the skin is intact, there will be a controlled uptake of water ions (Takle et al., 2015). Therefore, smoltification is closely related to the barrier status of the salmon skin (Figure 5) (Karlsen et al., 2018).

Figure 5: Atlantic salmon skin. The skin is closely related to the osmotic regulation, and therefore also the smoltification. Photo derived from https://nofima.no/verdt-a-vite/verdt-a-vite-om-fiskens-skinn/

6.3. FISH SKIN

The skin is the first barrier against external threats to the fish (Minniti et al., 2017). It is a vital organ that consists of several layers, including the epidermis, dermis, and hypodermis (Takle et al., 2015). Under these layers, there are both white and red muscles (Kryvi & Poppe, 2016).

6.3.1. EPIDERMIS

The epidermis (Figure 6) is the outermost layer of the salmon skin, consisting of several cell types along *stratum superficiale* (epithelial and mucus cells), *stratum spinosum* (mucus cells, nerve cells, club cells, ion cells and undifferentiated cells) and *stratum basale* (basal cells) (Takle et al., 2015). Two cell types with major protective and structural importance are mucus and keratocyte (epidermal) cells, respectively (Karlsen et al., 2012). The keratocyte cells are the primary cell type of epidermis (Sveen et al., 2020) and play a major role in wound healing (Sveen et al., 2019). These cells are bound together to adjacent cells by desmosomes and include vast interlinking filaments in their cytoskeleton (Kryvi & Poppe, 2016). These intermediary filaments provide great mechanical strength. Additionally, actin bands (microridges) (Sveen, 2018) are found on the upper layer of the epidermis cells. These provide a layer that the mucus easier can attach to (Kryvi & Poppe, 2016).

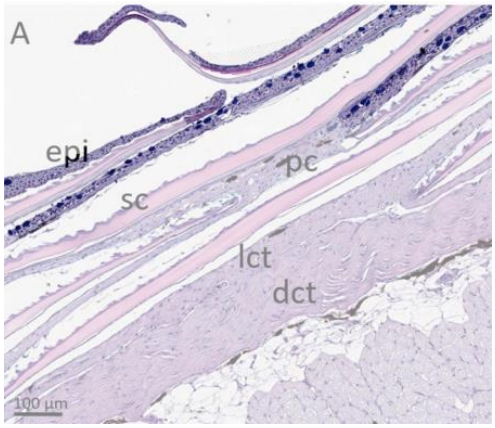


Figure 6: Histology of skin, highlighting the epidermis. Photo derived from (Sveen et al., 2021).

6.3.2. MUCUS

Mucus is the first protective barrier against external threats, protects the skin against mechanical damage and plays a role in osmoregulation (Micallef et al., 2012; Sprague & Desbois, 2021). Mucus primarily consists of proteoglycans and glycoproteins in addition to smaller amounts of sialic acid, antibodies (IgM), enzymes, and lysozymes (Easy & Ross, 2010; Hatten et al., 2001; Ross et al., 2000). The mucus is equally distributed on the skin surface, as macromolecules (secrete from mucus cells) swell and absorb water when given the ability. Mucus is produced by goblet cells around the skin (Valdenegro-Vega et al., 2014). These cells increase in size around the upper layer of the skin.

6.3.3. DERMIS

The dermis (Figure 7) can be found under the epidermis, with the basal membrane (*stratum basale*) in between (Takle et al., 2015). The dermis layers can be differentiated in two, based on consistency. The outermost layer (*stratum laxum*) consists of different cells, nerves, blood vessels, collagen fibres and scales. The inner layer in the dermis (*stratum compactum*) consists of more solid connective tissues, including vast collagen fibres that make the skin more elastic (Kryvi & Poppe, 2016).

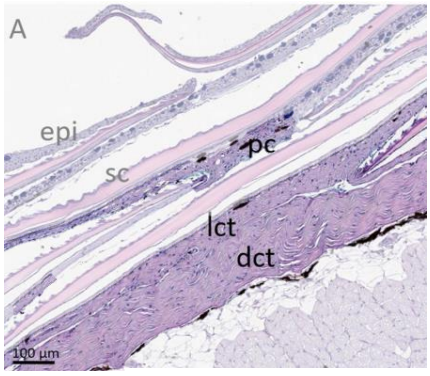


Figure 7: Histology of skin, highlighting the dermis. Photo derived from (Sveen et al., 2021).

6.3.4. SCALES

Besides collagen fibres, nerves and blood vessels, the dermis consists of scales (Figure 8). Scales are thin plates covered by the epidermis. The scales can vary in size and lie obliquely underneath the epidermis surface. The smooth transition between scales provides the body mechanical strength. Scales also protect the underlying tissues, meaning that losing them could lead to osmoregulatory problems (Noble et al., 2018). Teleost fish can have two different scales, including cycloid and ctenoid scales. Cycloid scales, found on Atlantic salmon (Peyronnet et al., 2007), have a globular shape with circuli rings that increase in numbers as the fish gets older (Thomas et al., 2019).

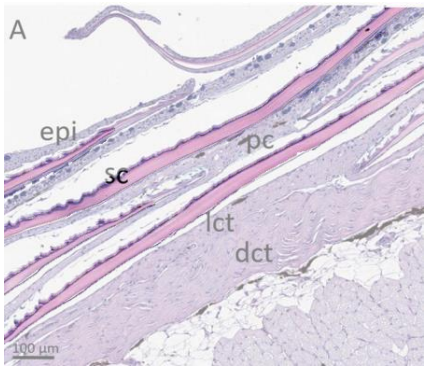


Figure 8: Histology of skin, highlighting scales. Photo derived from (Sveen et al., 2021).

6.3.4.1. SKIN PIGMENTATION

Pigmentation can also be found in the dermis. The pigment can be customized with the environment, providing a camouflage effect. This is due to chromatophores, which are different kinds of cells that contain pigment in their cytoplasm (Ligon & McCartney, 2016). The location of the pigment grains decides the intensity of the colour (Kryvi & Poppe, 2016).

6.3.4.2. HYPODERMIS

The hypodermis is the layer underneath the dermis (Takle et al., 2015). This innermost layer of the skin consists of lipid cells and loose connective tissue (Kryvi & Poppe, 2016).

6.3.5. SKIN OBSERVATION

Fish skin can be studied at macro, meso and micro levels. At macro level, it can be evaluated by visual perception. For instance, non-optimal vulnerabilities can appear as wounds, scale loss and bleedings. Thus, these are selected FISHWELL Operational Welfare Indicators (OWIs) that are used in the daily work at fish farms to evaluate salmon welfare (Noble et al., 2018).

Furthermore, skin can be evaluated by histology (meso level) and gene expression (micro level). Gene regulation is highly complex and displays whether a gene is transcribed. For instance, epigenetic marks can impact which genes that are expressed. This can, for certain genes, be altered by environmental and dietary factors. Thus, shifting from marine to plant ingredients could potentially affect the gene expression in Atlantic Salmon (Olsvik et al., 2011; Sahlmann et al., 2013).

6.3.6. ENVIRONMENTAL INTERACTIONS

Both biotic and abiotic factors can influence skin health. Abiotic factors, as handling, can lead to a significant amount of scale loss and mechanical damage to the skin. Also, biotic factors as smoltification can lead to loose scales (Noble et al., 2018). Furthermore, pathogenic microorganisms, salinity and water velocity could impact skin health. For instance, lower salinities after seawater transfer could be advantageous for smolts with a poor quality (ability to hypo-osmoregulate).

In addition to the environmental interactions on skin health, some functional feeds target functionality of the skin. Their composition is confidential, but zinc, omega 3 (Berge et al., 2019) and vitamin C are ingredients that could improve skin health (Jensen, L. B. et al., 2015b). However, these feeds are primarily developed for on-growing fish. Therefore, a better understanding of how feeds can influence the skin (defence mechanisms) will improve the work towards better skin health on juveniles as well (Jensen, 2015).

6.4. NUTRITION

Besides the potential of improving skin quality, it is essential to feed the fish correctly to maintain high digestibility, health, welfare, pellet quality (physical aspects), sustainability, and good product quality (Waagbø et al., 2001). Furthermore, species-specific and life stage-dependent nutrient needs to be fulfilled (Hardy, 2002).

Salmonids are carnivore species, meaning that they primarily eat organisms from secondary or tertiary trophic levels (Cottrell et al., 2021). This is evident during both freshwater and the saltwater stage, as their foraging behaviour is quite opportunistic (Dixon et al., 2012). Their native diet does, however, change throughout the lifecycle (Rikardsen & Dempson, 2011). During juvenile stages in freshwater, wild salmonids eat insects, crustaceans and amphipods. They tend to eat whatever floats by (Berntssen et al., 2003), which is advantageous as the food sources in rivers will change over time (Borgstrøm & Hansen, 2000). On the other hand, "*Atlantic salmon in the sea could be more selective due to their availability of marine food sources*" (Storebakken, 2020). They eat pelagic fish like herring and capelin, mesopelagic fish (lives deeper than 200 meters) as lantern fish, and small squids and crustaceans as krill and shrimps (Rasmussen, 2012; Storebakken, 2020).

Besides the change in diet, there is a change in body composition throughout the lifecycle. In juvenile salmonids, a large proportion of their body is composed of proteins. When the fish weighs less than 20 grams, the feed contains about 48% protein. Therefore, Atlantic salmon in juvenile stages have a relatively high need for essential amino acids and thus proteins. This protein amount (in feed) decreases to 34% when the fish has reached 1.5 kg (National Research Council, 2011), which changes the nutritional requirements accordingly. The salmon also have other important nutritional needs in specific life stages (Hardy, 2002). For instance, freshwater fish requires more iodine as iodine levels are low in freshwater. Furthermore, the need for biotin is likely greater during smoltification (Waagbø et al., 2001). These and several other conditions have to be considered when optimizing composition of commercial diets for different life stages (Hardy, 2002).

6.4.1. HISTORICAL DEVELOPMENT OF COMMERCIAL DIETS

The ingredients used in commercial diets have rapidly changed since the beginning of salmon farming in the 1970s. Marine ingredients have decreased while the plant ingredients have increased, both in protein and oils (Figure 9). Accordingly, the fish-in-fish-out ratio (FIFO) has been reduced over the years. In 1990, the feed consisted of 64.3% marine protein and no plant protein, while it in 2016 contained 14.5% marine protein and 40.3% plant protein. Correspondingly, the oils changed their origin from only marine sources in 1990 (24%) to 10.4% marine oils and 20.2% plant oils in 2016. Carbohydrate sources (binders) remained stable, and micro-ingredients (vitamin mix, mineral mix, astaxanthin and crystalline amino acids) *increased* during the same period (Aas et al., 2019). Consequently, the chemical composition of feed has changed. In the 1990s, the dietary lipids in salmon feed were between 8-22%, whereas it has increased to 35-38% nowadays.

The interaction between marine and plant ingredients is complex and could be affected by many aspects, including palatability, nutrient composition, digestibility, growth impact and cost of different feed ingredients (Pratoomyot et al., 2010; Tacon & Metian, 2015). Also, the availability of raw materials could be regulated by political restrictions and be affected by their competition with human resources.

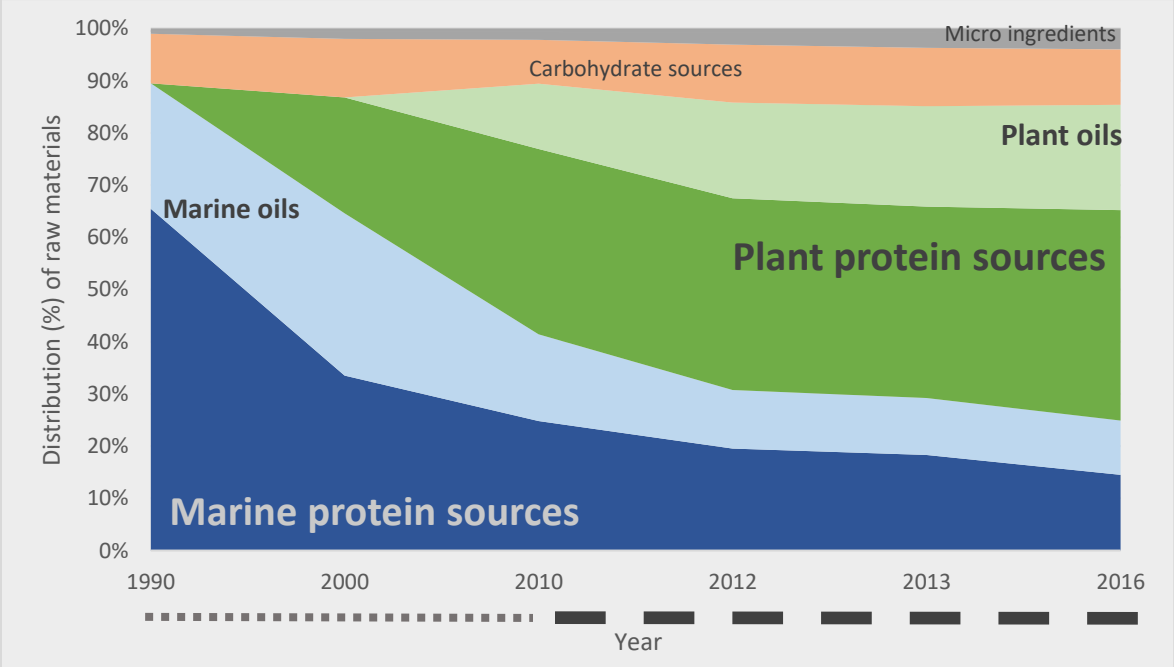


Figure 9: 100% Area chart showing the Norwegian development of raw material distribution (y-axis) of feed raw materials throughout selected years by the period of 1990-2016. Each of the coloured areas inside the diagram represent one source each. NB! Numbers are taken from uneven selected years, meaning that the interval between the years is smaller on the second half of the diagram, after 2010. The numbers are derived from (Aas et al., 2019)

6.4.2. MARINE INGREDIENTS

Marine ingredients used in aquaculture are primarily fishmeal and fish oil (Aas et al., 2019). Hence, 78% of the fishmeal today derives into aquaculture feed, whereof 15% to salmonids (EUMOFA, 2021). Fishmeal and fish oil are primarily processed from the Peruvian anchovy (*Engraulis ringens*), a pelagic forage fish species (Péron et al., 2010). Being a source of animal marine protein makes its amino acid balance like the predator/eater itself, and therefore a satisfactory source of protein for carnivores. Additionally, the Atlantic salmon is dependent on lipids from marine ingredients, and fish oil is exceptionally rich in the healthy omega 3 EPA and DHA (long-chain PUFAs). Atlantic salmon have a limited capacity to synthesise the essential fatty acids (EFA) 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid), which makes it essential to supplement their diet with EPA (20:5n-3) and DHA (22:6n-3) (Waagbø et al., 2001). Also, long-chain fatty acids have a low melting point. Thus, they provide fluidity of the cells' membranes, which is essential for cold-water marine species.

Despite the beneficial nutritional perspective of marine resources, fishmeal and fish oil consumption cannot increase further. The gap between the omega-3 supply and demand is critical (Hamilton et al., 2020), and an increasing amount of wild-caught fish are used directly for human consumption. Furthermore, the world fish meal and fish oil production are majorly dependent on the Peruvian anchovy fisheries and the amounts caught from these sources vary due to structural changes and El Niño (temperature oscillations) (Moron et al., 2019). Moreover, using ingredients from high trophic levels is less sustainable and can lead to a higher accumulation of harmful PCBs in the salmon we consume. There will consequently be a need for even more sustainable and alternative feed ingredients to fulfill marine resources' demand.

Today, fish meal and fish oil cannot be entirely replaced with alternative marine ingredients. However, some amounts could be replaced by by-catch/by-products, mesopelagic fish and alternatives from lower trophic levels (copepods, krill, amphipods). Also, other organisms could probably replace marine components in favour of lipid and proteins in the future. Examples are bacteria, yeast/fungi, micro-algae and insects (BELLONA, 2021). These alternatives are likely to be further developed to improve a more sustainable circular bioeconomy, utilising sources that are not used for human consumption today.

6.4.3. PLANT INGREDIENTS

The overall main feed ingredient in Norwegian aquaculture farming of Atlantic salmon is soy protein concentrate (SPC). In 2016, it represented 19% of the ingredients used. After soy, wheat gluten (9%) and corn gluten (3.6%) were the second and third major sources to plant protein. In terms of plant oils, rapeseed and camelina oil dominated, yielding a total amount of 19.8%. Also, carbohydrate sources as wheat showed a significant volume (8.9%) (Aas et al., 2019).

The rapid use of terrestrial ingredients from plants has almost made the Atlantic salmon diet completely herbivore. However, this replacement seems to have a minor effect on the fish in terms of health, except its negative inflammatory impact on the distal intestine (Waagbø et al., 2013). Additionally, marine diets for salmonids are dependent on a certain amount of terrestrial ingredients as wheat to be used as pellet binders (Storebakken et al., 2000). On the other hand, plant ingredients contain more harmful antinutrient factors (ANF), but their impact could be reduced by technical processing (Francis et al., 2001). Also, the rapid use of plant ingredients is more dependent on using several plant sources to equalise the balance of amino acids and lipids (Espe et al., 2006). For instance, vegetable oils lack the long-chain omega-3 fatty acids the salmon is dependent on. However, the advancements in biotechnology have shown promising results for producing terrestrial-based omega-3 through genetically modified plants (with omega-3 genes) (Napier et al., 2019).

The main driver for using plant ingredients in aquafeeds has been their low cost. Also, plant ingredients are not finite as fish meal and fish oil. However, plant ingredients are resource-demanding due to their high use of freshwater, land areas, deforestation, and competition with ingredients for human consumption (humans could directly use ex. soy) (Aas et al., 2019). Therefore, a sufficient Life cycle assessment (LCA) must be performed to assess the sustainability of different feed ingredients (Smetana et al., 2016; Taelman et al., 2013). Studies have shown that we can not only rely on the plant ingredients used at present. Therefore, feed ingredients are likely to change in the future (BELLONA, 2020).

7. MATERIAL AND METHODS

7.1 FISH MATERIAL AND EXPERIMENTAL DESIGN

The data was collected on Nofima´s research station for sustainable aquaculture in Sunndalsøra, Mid-Norway (Figure 20). The experimental trial was approved by the Norwegian Animal Research Authority (NARA) and conducted following regulations concerning experiments and procedures for live animals in Norway.

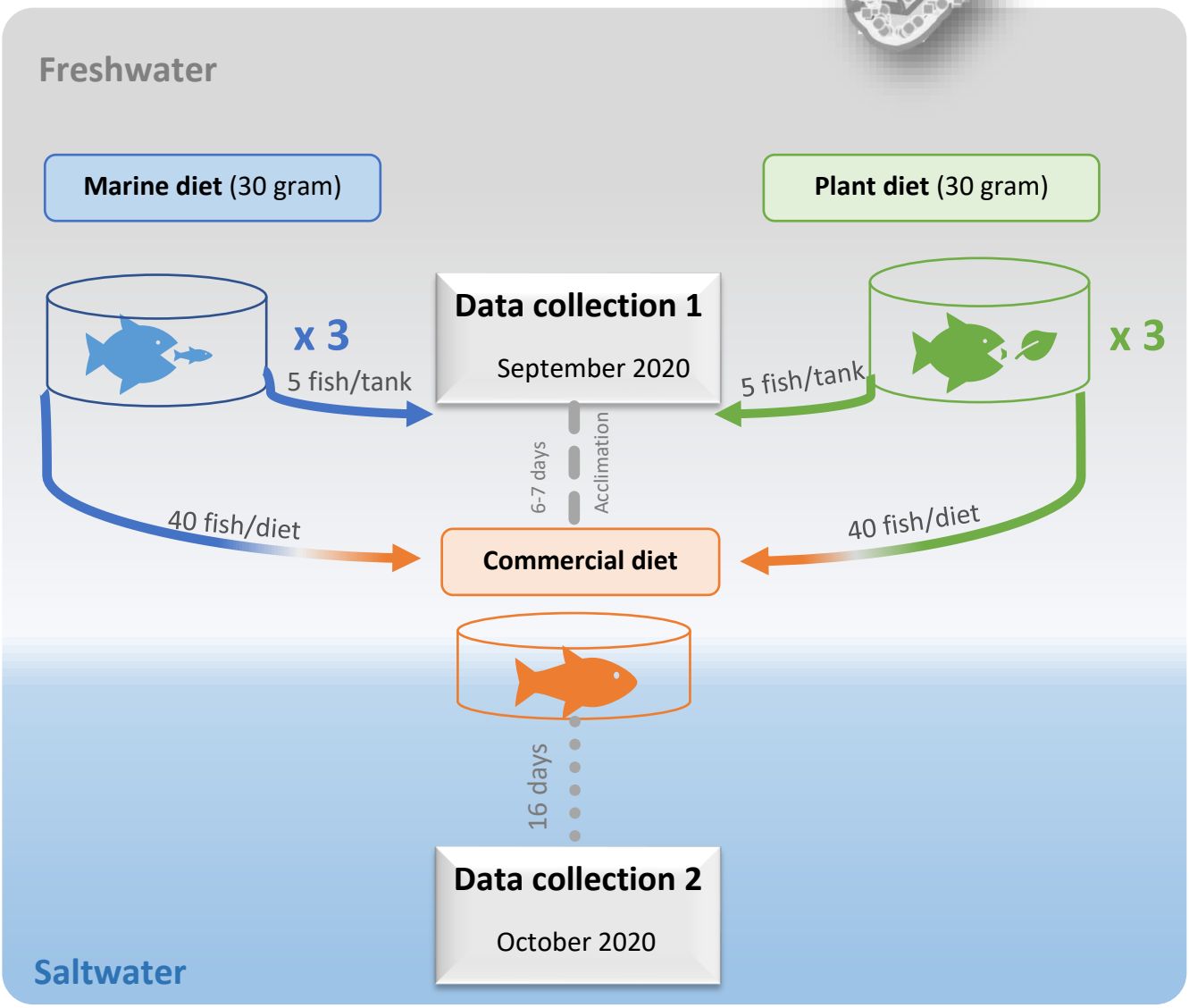


Figure 20: Illustration of the experimental design, from hatching (Dec. 2019) until the second data collection (Oct. 2020). The map in the right corner displays the location of the site where the experiment occurred (Barentswatch).

A batch of eyed Atlantic salmon (*Salmo Salar* L.) eggs from the same genetic origin (Bolaks) was hatched in Sunndalsøra, December 2019. Start feeding began when the fish had reached fry stage after 861 d°, in January 2020. Then, 110 fish (used in this study) were randomly placed into six circular tanks and kept in a flow-through system. The fish were PIT-tagged when they reached 30g on average and distributed to eight 500L tanks fed either a plant-based diet or a diet based on marine ingredients (triplicate tanks per diet) during the period July 7th to September 16th 2020. Smoltification was stimulated by exposing the fish to a light regime mimicking a natural summer- winter- spring daylength signal (12:12) from July 13th. August 26.-27th, the fish was vaccinated (average weight 83g). September 16.-17th., five fish from each tank were sampled for analyses, before 40 random fish from each diet were transferred to a 6360L tank September 22nd, where they were farmed together and fed a standard commercial diet (Skretting, Stavanger, Norway). After 16 days in saltwater, all 80 fish were sampled for analyses. The fish groups sampled in freshwater are termed Freshwater Marine (n=15) and Freshwater Plant (n=15), whereas the fish groups sampled in saltwater are termed "Saltwater Marine" (n=40) and "Saltwater Plant" (n=40). At each sampling point, fish were weighted, length was registered, and fish were photographed for subsequent determination of FISHWELL scale loss scores (Operational welfare indicators, OWIs). Additionally, skin samples from 60 and 47 fish were fixed in formalin for subsequent histology and gene expression analyses, respectively.

7.2. PRODUCTION ENVIRONMENT

In freshwater, the tanks had an average temperature of 10.1°C and a fish density of 21.6 kg/m^3 at saltwater transfer. In saltwater, the tanks had an average temperature of 10.6°C and a fish density of 3 kg/m^3. The mortality rate was 0.56-1.25 for the whole batch (Figure 11).

The fish were smoltified through 12L:12D light manipulation. This method, combined with the earlier mentioned water temperature, caused smoltification as 0+ (the same year as hatching). A salinity test was performed the September 15th, 64 days after the light treatment started.

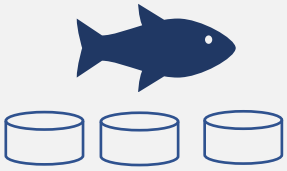
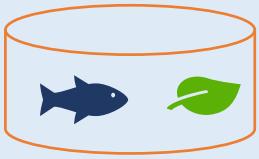
Production parameters - Average values			
	FW Marine 	FW Plant 	SW Commercial diet 
Temperature (°C)	10.1 (min 8.7-max 12.3)	10.1 (min 8.7-max 12.3)	10.6 (min 7.3-max 13.1)
Fish density (kg/m^3)	21	21	3.0
Salinity (‰)	0	0	33.2 (min 31.7- max 34.1)
Tank volume (liter)	500	500	6360
Mortality (%)	1.11	0.56	0.0

Figure 11: Overview of production parameters of Atlantic salmon fed a marine based diet (Marine) or a plant based diet (Plant) in freshwater (FW; triplicate tanks per diet). In seawater (SW) all fish were farmed commonly and fed a commercial diet.

7.2.1. FEED REGIME

In freshwater, feed was offered for 42 seconds with intervals of 900 seconds. In saltwater, feed was offered for 10 seconds with intervals of 720 seconds. No distinct foraging behavior was observed during the experiment.

7.3. DIET

7.3.1. FEED INGREDIENTS

The feeds were produced at Nofimas Feed Technology Center. Figure 12 (Appendix 1) displays the distribution of feed ingredients (%) in both test diets. The marine diet did primarily consist of fish meal (61%), followed by wheat (20.7% (pellet binder) and fish oil (15.4%). The plant diet primarily consisted of SPC (soy protein concentrate, 34.6%), wheat gluten (22%), and rapeseed oil (20.4), supplemented by smaller amounts of corn gluten (10%) and wheat (8.4%). In addition, both feeds contained <5% other supplemented ingredients (the grey part of bars in Figure 12). These supplements included ingredients that are necessary or preferred in smaller amounts, such as vitamin and mineral premix.

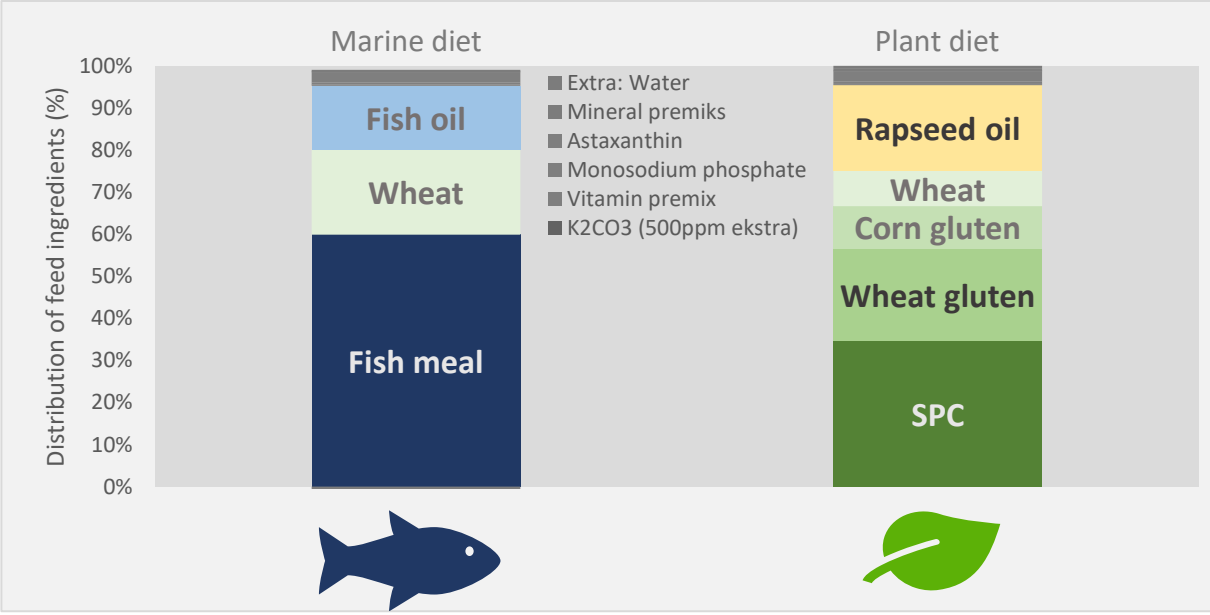


Figure 12: 100% stacked column chart, showing the distribution of feed ingredients (%, y-axis) in the marine (left bar) and plant test diets (right bar). The grey/top part of the bars indicate several ingredients, described between the bars (marked in grey).

Detailed information on the *origin* (country of origin, factory etc.) of each raw material can be found in Appendix 2.

7.3.2. CHEMICAL COMPOSITION

Figure 13 shows the composition (%) of nutrients in the experimental test diets. Both the marine and the plant diet primarily contained protein, followed by lipids, starch and ash. The marine diet consisted of 46.1% protein, 23.6% lipids (including 14.3% EPA + 17.8% DHA), 12.6% starch and 9.7% ash. The plant diet contained 46.8% protein, 24.9% lipids (including 0.08% EPA + 0.17% DHA), 9.3% starch and 5.1% ash.

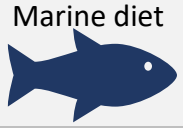
Calculated chemical feed composition (%)		
	Marine diet 	Plant diet 
Protein	46.1	46.8
Lipid	23.6 (14.3 EPA, 17.8 DHA)	24.9 (0.08 EPA, 0.17 DHA)
Starch	12.6	9.3
Ash	9.7	5.1
Water	9.2	9.2
Energy, MJ/kg	22.1	20.8

Figure 13: Table showing the chemical distribution (%) of nutrients in the marine and plant test diets.

7.4. LEVELS OF DATA SAMPLING

The skin of each fish was studied at macro-, meso- and micro-level (Figure 14). Respectively, these levels indicate morphological welfare indicators (macro-level), histology (meso-level) and gene expression (micro-level).

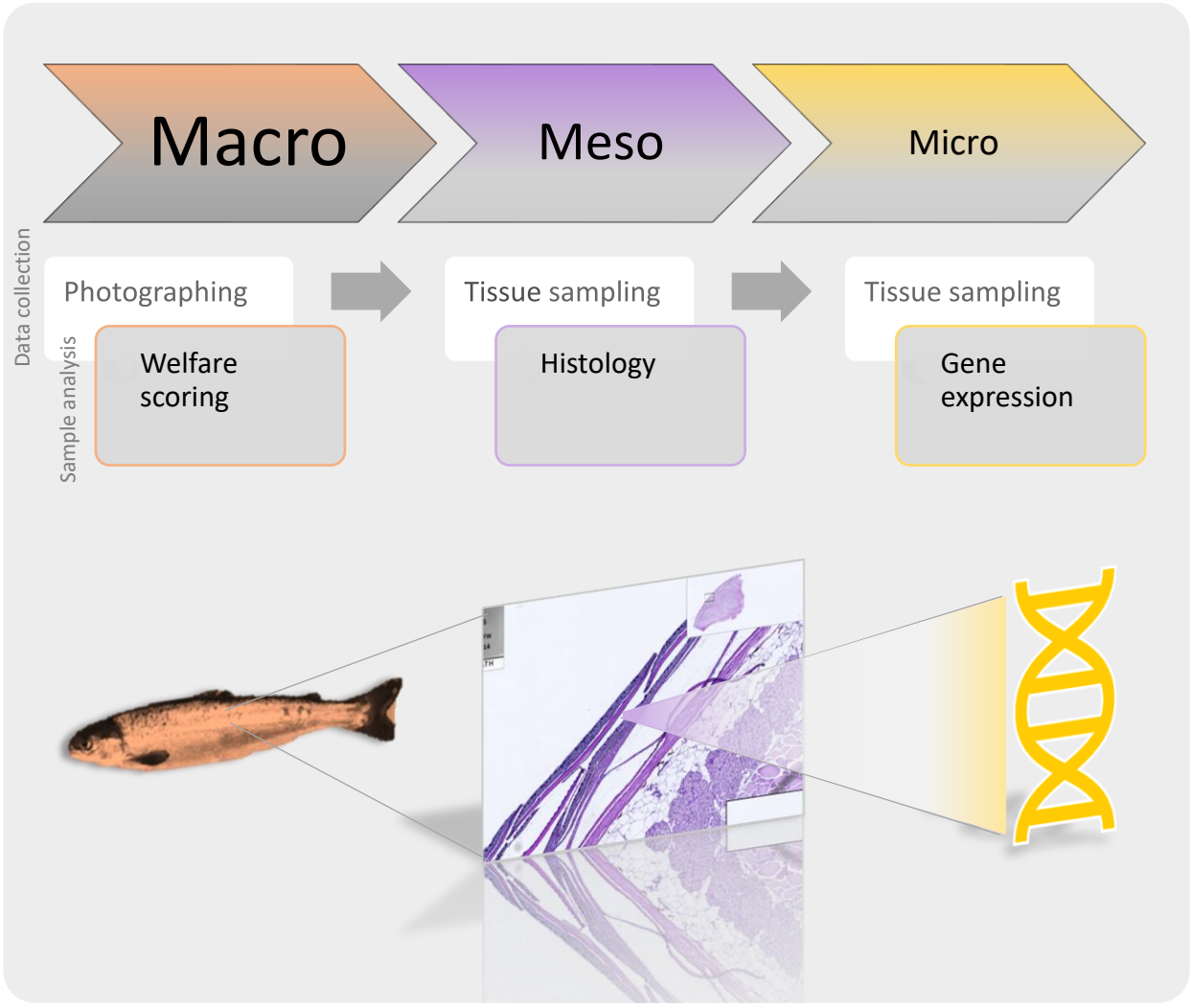


Figure 14: Illustration of data sampling levels, including macro- (welfare scoring), meso- (histology) and micro-level (gene expression).

7.5. Data collection

The fish were sampled from each tank using a catch net and further anesthetised to death in a bucket with water and Benzoak® before measuring the length and weight of each individual. The data collection consisted of three main steps (Figure 14): photographing (Appendix 3), tissue sampling for gene expression (Appendix 4) and tissue sampling for histology (Appendix 5). Each method is explained in numeric order below their representative illustrations.

7.6. Analyzing samples

The different analyses includes welfare scoring of the fish by pictures (Figure 15), gene expression of the skin (Appendix 6) and microanatomy of the skin studied by histological methods (Figure 17).

7.6.1. Welfare scoring

The photo of each fish was used for evaluating scale loss using categorical scores between 0-3. Score 0 represents no scale loss, while score 3 represents severe scale loss (Figure 15).

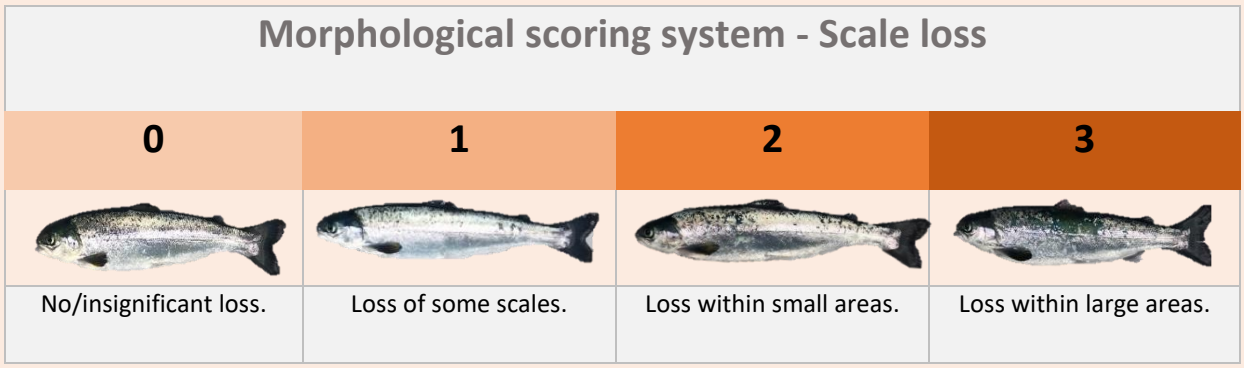


Figure 15: Morphological scoring system for Atlantic salmon, describing and illustrating the degree of scale loss.

7.6.2. MICROANATOMY STUDY

Sixty tissue samples (one from each fish, equally distributed in terms of diet and salinity) from the above-mentioned sampling (step 8.-10.) were sent to the Norwegian Veterinary Institute (NVI) and stained according to the AB-PAS-method (Alcian Blue – Periodic Acid Schiff), sectioned and scanned.

7.6.2.1. PREPARATION

When receiving the images, further preparation for the microanatomy study included understanding and downloading the picture analysis program Aperio ImageScope (Version 12.4.5008).

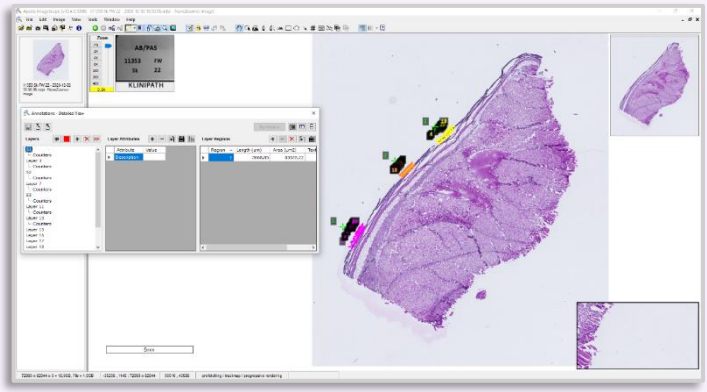
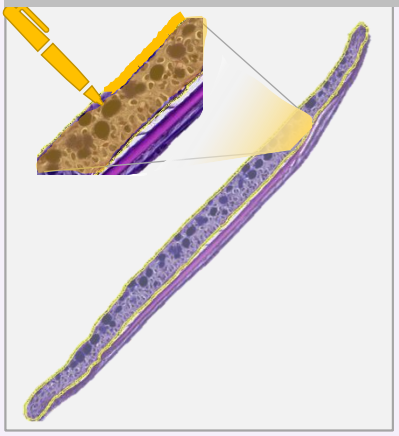


Figure 16: Screenshot from Aperio ImageScope, which was used to analyze the histology pictures. Each sample were delivered like the one demonstrated above.

7.6.2.2. METHOD

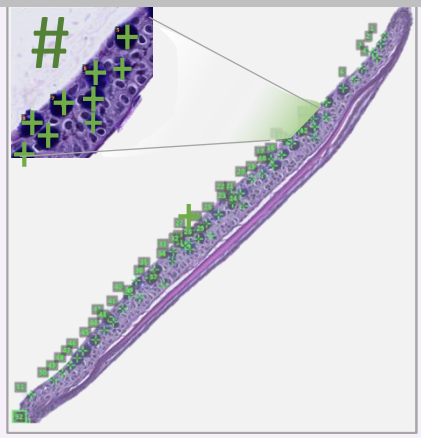
The method included studying epidermis layers by looking at 3 representative epidermis layers from each fish/picture/sample (Figure 16). All the observations were stored and structured by utilizing the annotation tool. The following further steps were manually performed in numeric order for each of the 60 samples (Figure 17):

1. Find Layer Area



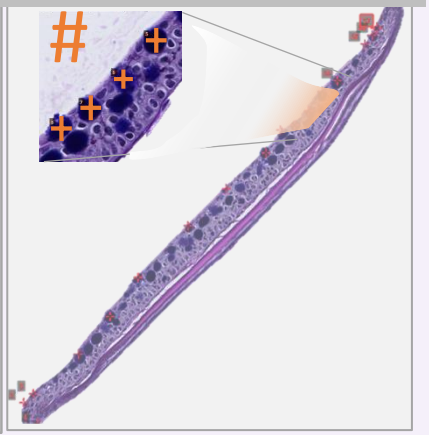
Use the pen tool to mark along the edge of one epidermis layer. Note the result (um^2) and follow the further steps (2.-5.) on the same epidermis layer.

2. Count mucus cells



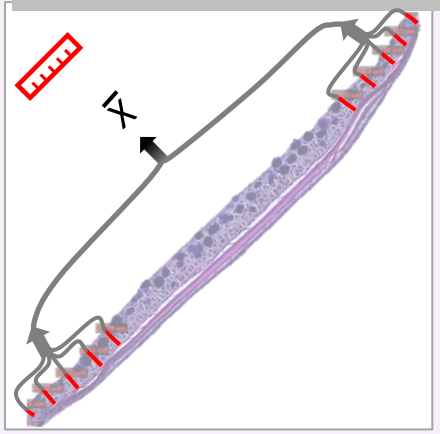
Use the counter tool to count all mucus cells on the epidermis area. Note the result (n).

3. Count external cells



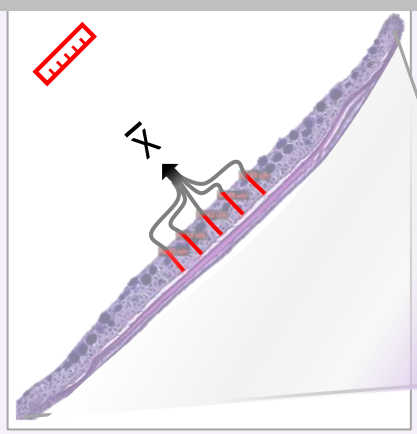
Use the counter tool to count the outermost/apical cells. Note the result (n).

4. Find End-thickness



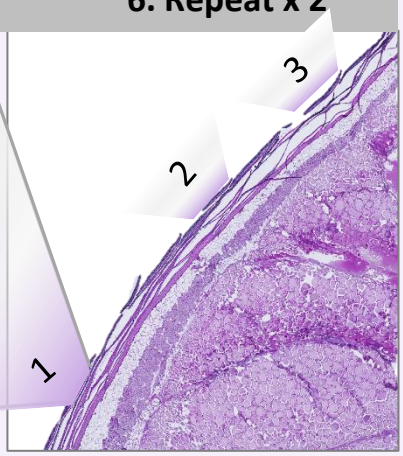
Use the ruler tool to find the width of the 20% outermost part of the epidermis layer by marking 5 lengths from both sides. Note these 10 observations and find the average value (um).

5. Find Mid-thickness



Use the ruler tool to find the width of the 20% mid-part of the epidermis layer by marking 5 lengths. Note these 5 observations and find the average value (um).

6. Repeat x 2



Repeat step 1.-5. Twice for two *other* epidermis layers on the same picture (same fish). This will result in data from 3 mucus layers on each sample/picture/fish.

Figure 17: Method for microanatomy study of epidermis, displayed in numerical order.

7.6.2.3. DATA TREATMENT

All observations were stored in Excel. Then, the mucus ratio for all mucus cells (Figure 17, step 2) and apical mucus cells (step 3) was found by the following formula:

$$Mucus\ Ratio = \frac{Mucus\ cells}{Mucus\ layer\ area}\ x\ 1000$$

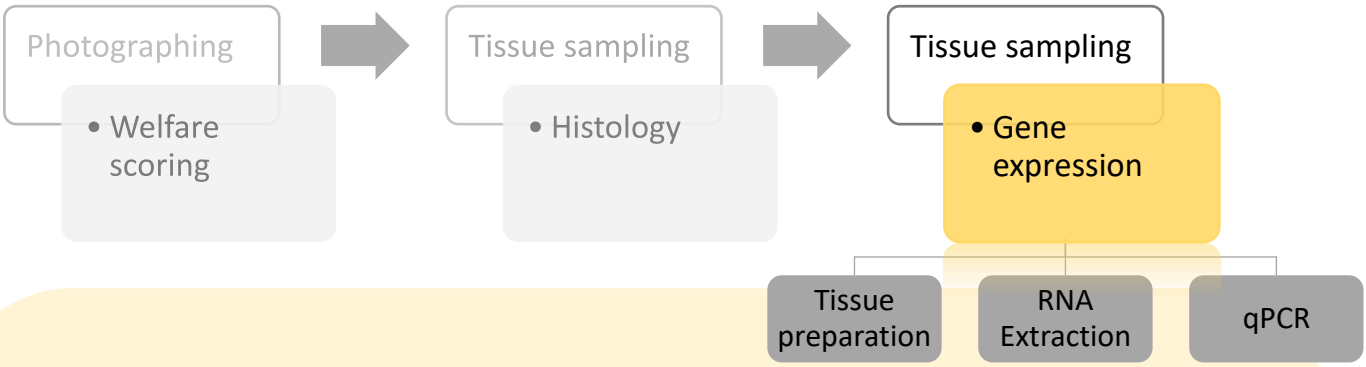
This resulted in two additional variables for each epidermis layer.

The histology measures' overall results (Figure 17) generated in 3 replications/repeated observations for each sample/picture/fish. Consequently, the average value for each variable was found (Figure 18) and used in the statistical analyses.

Fish$_i$																				
Epidermis layer 1							Epidermis layer 2							Epidermis layer 3						
Area	Cells	Apical Cells	Cell Ratio	Apical Cell Ratio	End-thickness	Mid-thickness	Area	Cells	Apical Cells	Cell Ratio	Apical Cell Ratio	End-thickness	Mid-thickness	Area	Cells	Apical Cells	Cell Ratio	Apical Cell Ratio	End-thickness	Mid-thickness

Fish$_i$						
Epidermis layer $\bar{x}$						
Area	Cells	Apical Cells	Cell Ratio	Apical Cell Ratio	End-Length	Mid-length

Figure 18: Table showing how 3 replications for each fish generated in one average value for each variable.



7.6.3. Gene expression

Expression of three selected stress related genes was studied; *MMP9*, *iNOS* and *Hsp70*. After tissue sampling, several steps were followed to obtain the result, including tissue preparation, RNA extraction and qPCR. In total, samples from 12 individuals from each of the four fish groups were sampled, except from "Freshwater Plant", which had 11 (in total 47 samples).

7.6.3.1. Method: tissue preparation

Each tissue sample had to be prepared for gene expression by following the numeric order in Appendix 6.

7.6.3.3. Method: RNA extraction

After preparing the tissue samples, RNA could be extracted from the epidermis. RNA extraction was performed automatically by a robot called Biomek Agencourt RNA Advance Tissue Kit. First, the prepared tissues were thawed in a heated Termarks cupboard at 37 degrees. Afterwards, RNA was extracted from the samples by following this robot's protocol.

7.6.3.4. Checkpoint: control RNA ratio and amounts

After RNA was extracted, its quality (260/280 ratio (purity indicator) and amounts) was controlled on a representative quantity of RNA samples (here: 14 samples) (Appendix 7). Perform this step by following the NanoDrop 1000 procedure and further test it by using an Agilent 2100 Bioanalyzer. If the result is acceptable, proceed to the next step.

7.6.3.5. METHOD: QPCR

qPCR was performed to quantify gene expression. Due to covid-19 and lab restrictions at Nofima, the quantitative reverse transcription-PCR (qPCR) was completed by Nofima engineers. Therefore, only RNA extracted from the epidermis was used. Briefly, samples were treated by DNase I (Invitrogen), and synthesis of the cDNA was carried out using TaqMan® Reverse Transcription Reagents (Applied Biosystems™) utilising the random hexamer protocol. Real-time PCR amplification was performed in 384-well plates using QuantStudio5. The reaction was performed using Power SYBR green PCR master mix with 0.5 µM of each forward and reverse primers and 2.34 µl of template cDNA. Expression of the target genes *mmp9*, *iNOS*, and *hsp70* were normalised to the level of EF1a, and values transformed into average expression in log2 scale. Each biological sample was run in three replicates for all four genes to ensure reproducibility. The amplification efficiency of the qPCR reaction for the gene primers had efficiency values between 89 and 105%. Gene-specific qRT-PCR amplification was verified by melting curve analyses.

7.6.4. DATA ANALYSIS

The data sampling resulted in a dataset with several continuous response variables, including weight, condition factor ($\frac{100\ x\ Weight\ (gram)}{Length(cm)^3}$), histology measures (mucus cells, apical cells, area, ratio, end-thickness, mid-thickness) and gene expression measures (MMP9, iNOS, Hsp70). Their explanatory variables were diet (Marine or Plant) and salinity (FW or SW), resulting in four different groups (FW Marine, FW Plant, SW Marine, SW Plant). In contrast, scale loss is a discrete variable that had diet and salinity as explanatory variables.

All variables were analysed in RStudio, Microsoft Excel and SAS. In Excel (Version 16.45), the dataset was explored by making bar charts, column charts, scatterplots, box plots and dotplots. Also, average values and standard errors were found, and major outliers in the dataset were deleted. Further on, the statistical analyses were performed in RStudio (Version 1.4) and SAS® (Version 9.4). The significance level in all tests was $p < 0.05$.

The Shapiro-Wilk Test was used to determine if the predictor variables were normally distributed (Appendix 8), and residual plots were made to make sure the variance was homoscedastic. The normally distributed variables with adequate residuals were tested with ANOVA. If the ANOVA test showed significant differences in the data ($p < 0.05$), a Tukey HSD test was performed to determine which group means were significantly different from each other. Fish weight was used as a covariate when it had a significant impact on the result. Additionally, Pearson's correlation plot was performed for all continuous variables, overall and divided by group (FW Marine, FW Plant, SW Marine, SW Plant). In contrast to the continuous variables, the discrete variables (scale loss as response variable, diet and salinity as explanatory variables) were tested by the Chi-Square test.

7.6.5. METHODOLOGICAL CONSIDERATIONS

Nutritional studies require standardised environments since the variance between groups should be caused by the feed(s) rather than the environment. Throughout this experiment, the environment was similar for both diets, and the fish were reared by trained personnel in a research facility. Therefore, the environmental influence is assumed to be highly eliminated, and the requirements for a match pair design are sufficiently met. Also, repeated data collections provide a representative understanding of the fish both before and after seawater transfer. Additionally, two fish were used for practice purposes before the data collection.

7.6.5.1. SOURCES OF ERROR – WELFARE SCORING

In general, welfare scoring is performed on the site while the fish is alive (Noble et al., 2018). However, this leads to unfavourable air exposure and makes it hard to compare the fish later. Therefore, Trovaag (2019) suggested methodological improvements that were implemented in this experiment. This included photographing the fish and using the pictures for evaluating the scores later. Also, further improvements included using a photography studio light box, which decreases reflection from clothes and pictures can be taken from a fixed position. These refinements strengthen the validity of this project through more standardised photographing. However, the picture evaluation method is subjective since the welfare results are evaluated visually. Therefore, further methodological improvements could include evaluation using objective image analysing methodology (new technology). This would decrease the human bias (Pronin, 2007), thus making the results more reliable. Otherwise, sources of error could be related to the harvesting method. Ideally, the fish would not be handled immediately towards the scoring (here: towards the photographing) as handling could lead to scale loss (Noble et al., 2018). Additionally, the amounts of fish studied were unequal. Thirty individuals were studied in freshwater, whereas 80 individuals were studied in saltwater. Thus, the results in saltwater are more valid.

In general, scale loss is a welfare indicator covered in both FISHWELL and RSPCA welfare standards (Noble et al., 2018). Therefore, scale loss was evaluated as a representative skin macro parameter in this experiment.

7.6.5.2. SOURCES OF ERROR - HISTOLOGY

The skin's surface is quite sensitive regarding abrasion (physical trauma causing mild abrasion of the external surface), which could be challenging for the samples. Thus, this is the primary error source for histology. Also, it was not always clear how many cells should be counted (some were difficult to distinguish), and the number of cells varied highly between replicates. Similar to the scale loss method, histology could therefore be evaluated through artificial intelligence (Sveen et al., 2021). However, evaluation of several repeated samples improve the validity in this trial, especially in terms of epidermal thickness. Mid- and end-thickness results are considered as highly repeatable since each length is measured 900 and 1800 times, respectively.

The amount of mucus cells differs between body regions (anterior/posterior, dorsal/ventral) (Takle et al., 2015). Thus, the localization of the biopsy is of high importance – both when evaluating the standardization of the method in this project *and* when comparing the number of mucous cells in this study with other studies and species. In this experiment, the skin biopsy for histology was performed at a fixed position (posterior to the dorsal fin, above the lateral line), in accordance with (Karlsen et al., 2018).

Mucous cells and keratocytes are the most numerous cell types in the epidermis of Atlantic salmon (Sveen et al., 2019). However, the epidermis layer consists of several cell types, and no cells in this study have been identified by molecular cell markers. Therefore, it is unclear *which* specific cell type the diet could influence – even though the cells counted are likely mucous cells or keratocytes.

7.6.5.3. SOURCES OF ERROR – GENE EXPRESSION

As previously mentioned, skin biopsies could be at high risk of contamination during sampling (Taylor et al., 2019). However, the graphs showed promising results regarding RNA quality (purity and amounts). Also, the results could be impacted by the reference genes used since it is necessary to use reference genes with stable expression during specific environmental conditions (Kortner et al., 2011). Furthermore, the standard error for all tested genes was extensive in all groups, which is challenging due to individual variability (Karlsen et al., 2018). Thus, a higher number of samples would be preferred to improve the samples' precision.

8. RESULTS

8.1. FISH SIZE

8.1.1. WEIGHT

The marine groups had a significantly higher weight than the plant groups ($p < 0.0001$) (Figure 19). SW Marine had the highest average weight (118 *± 3.4*), followed by FW Marine (111.7 *± 3.6*), SW Plant (89.3 *± 2.0*) and FW Plant (81.3 *± 2.9*). The fish weight increased numerically by 6-10% after transfer to saltwater, but the weight increase was not significant ($p = 0.129$).

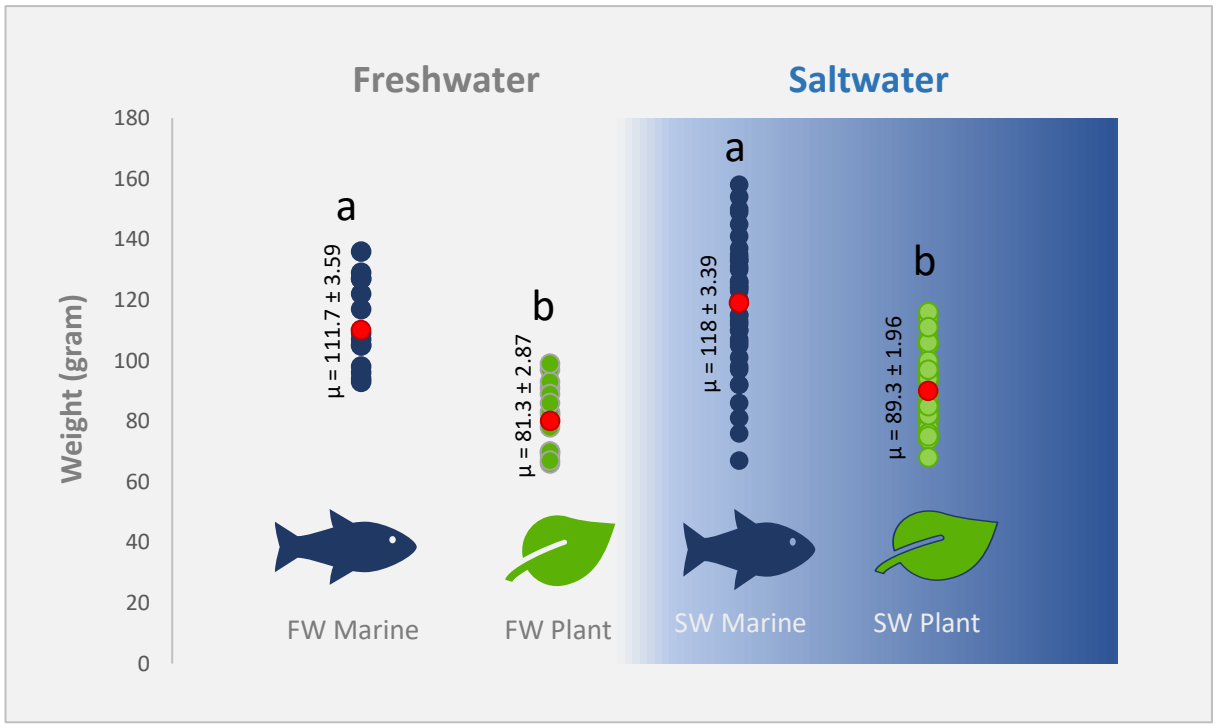


Figure 19: Dot plot showing the individual body weight distribution (gram, y-axis) of Atlantic salmon smolts, separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant), resulting in the four following groups: "FW Marine", "FW Plant", "SW Marine" and "SW Plant". The index with the weight closest to the given group's average value is highlighted in red. Average values ± standard error (SE) for each group is vertically shown beside each group. Also, the letters above each group indicate significant differences between the groups. The significance level is $p < 0.05$.

8.1.2. CONDITION FACTOR

The freshwater groups had a significantly higher condition factor than the saltwater groups ($p < 0.0001$) (**Error! Reference source not found.**0). The FW Plant group had the highest average condition factor (1.29 *± 0.02*), followed by FW Marine (1.28 *± 0.03*), SW Marine (1.14 *± 0.01*) and SW Plant (1.13 *± 0.01*). No significant difference was found in terms of dietary treatment ($p = 0.276$).

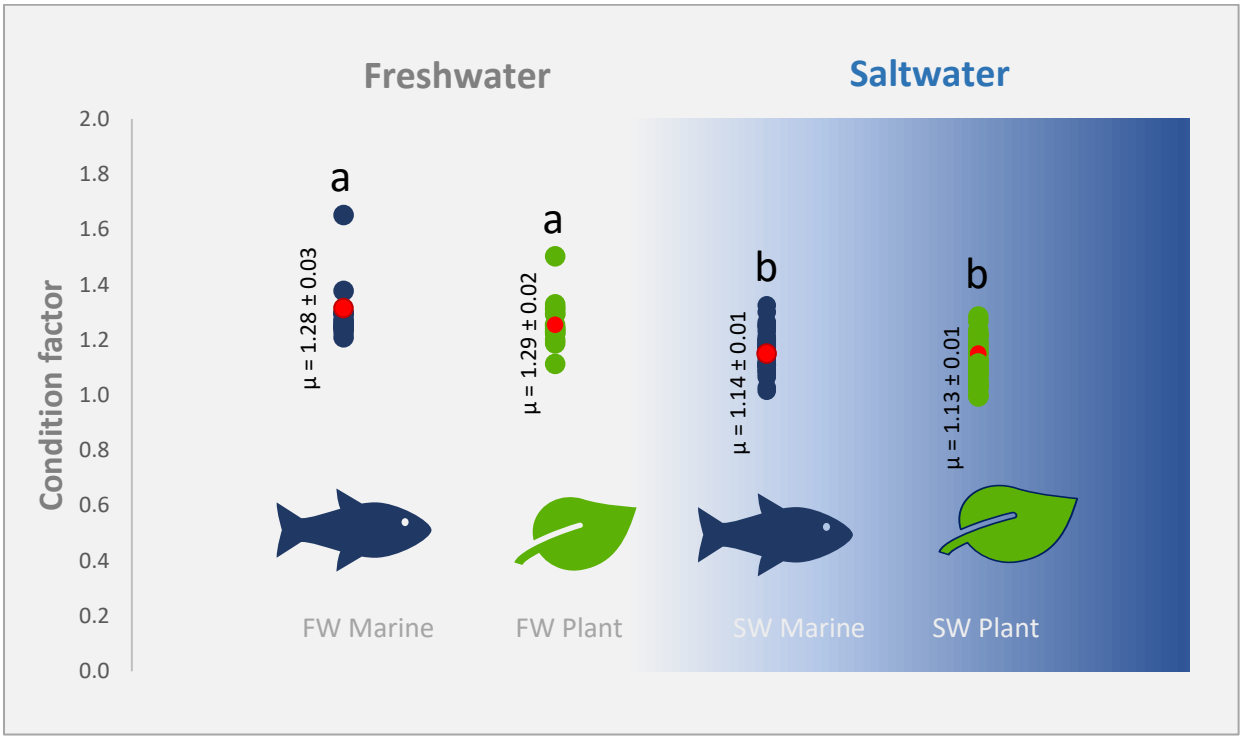


Figure 30: Dot plot showing the individual condition factor distribution (CF, y-axis) of Atlantic salmon smolts, separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant), resulting in the four following groups: "FW Marine", "FW Plant", "SW Marine" and "SW Plant". The index with the CF closest to the given group's average value is highlighted in red. Average values ± standard error (SE) (adjusted for fish weight) for each group is vertically shown beside each group. Different letters above each group indicate significant differences between the groups. The significance level is $p < 0.05$.

8.2. MACRO-LEVEL: WELFARE SCORING

Most fish were categorized into scale loss score 0 (46%) or score 1 (46%) (Figure 21). Score 0 was most common in freshwater, while score 1 was most common in saltwater. 47% of the freshwater fish had scale loss (defined by score 1 or higher), whereas the percentage of fish with scale loss increased to 61% after transfer to salt water. According to the chi-square test, this increase in scale loss was, however, not significant ($p = 0.21$). There was no dietary effect on scale loss ($p = 0.21$).

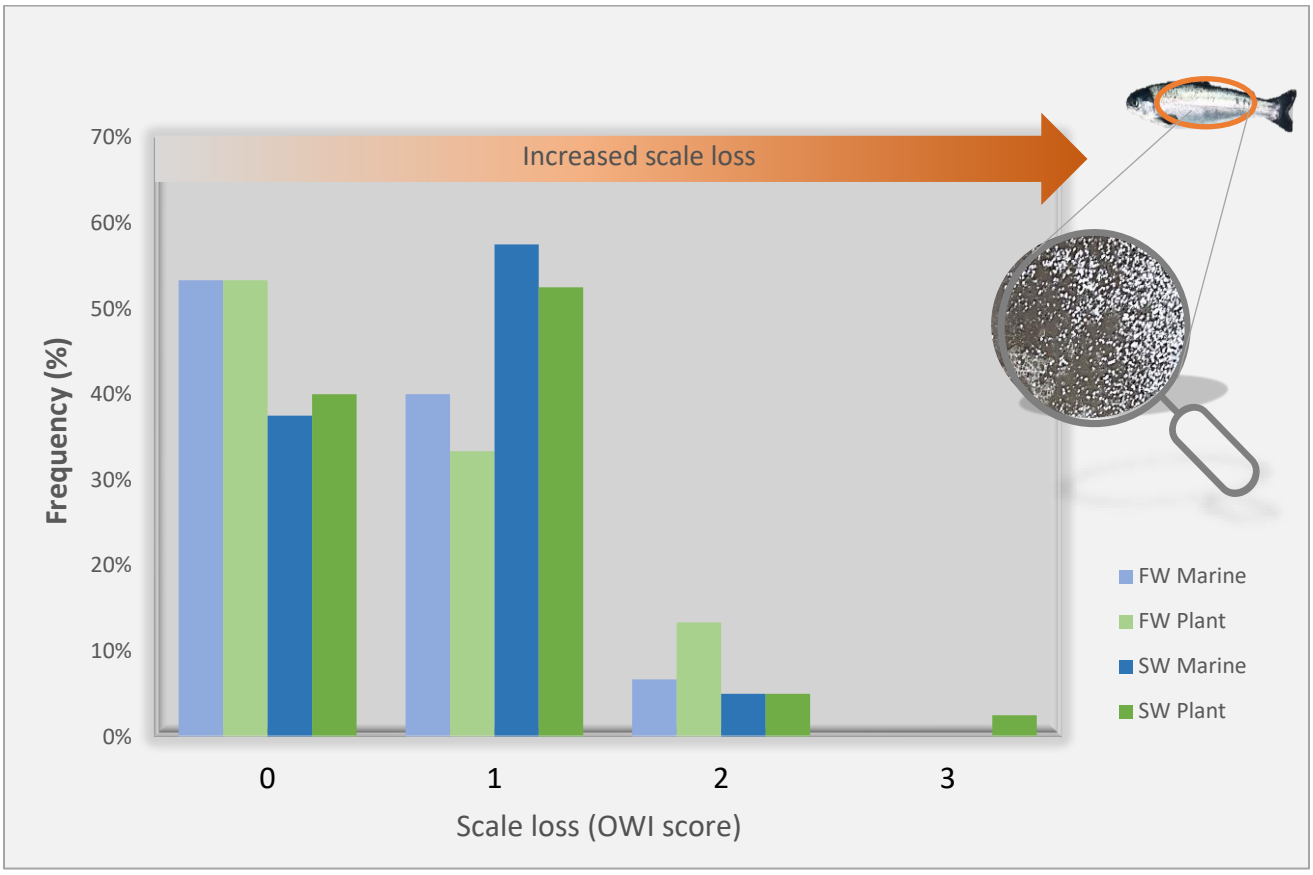


Figure 21: Grouped bar chart showing the distribution (%, y-axis) of categorical OWI (Operational Welfare Indicators) scale loss scores (0-3, x-axis) of Atlantic salmon smolts, separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant), resulting in the four following groups: "FW Marine", "FW Plant", "SW Marine" and "SW Plant". The severity increases according to the score, indicating that score 0 means no scale loss and score 3 means loss of scales within large areas.

8.3. MESO LEVEL: HISTOLOGY

8.3.1. MODEL ASSUMPTIONS

According to the Shapiro Wilcoxon Test, all seven histology variables except from mucus area and external mucus cells, were normally distributed (Appendix 8). However, the residuals from each of the variables were homoscedastic.

Overall, five histology variables (mucus ratio ($p < 0.0001$), epidermis area ($p = 0.0021$), amount of mucus cells ($p < 0.0001$) epidermal end-thickness ($p = 0.0315$) and epidermal mid-thickness ($p < 0.0001$)) tested by one-way ANOVA differed significantly between groups, either in terms of diet or salinity or both ($p < 0.05$). Also, three out of these five 5 showed an interaction between fish weight, meaning that the results and average values for epidermis area ($p = 0.0021$), amount of mucus cells ($p < 0.0001$) and epidermal mid-thickness ($p < 0.0001$) were corrected for weight differences.

8.3.2. MUCUS RATIO

There was a significantly higher amount of mucus cells/1000 µm^2 area in freshwater than in saltwater ($p < 0.0001$) (Figure 22, purple bars). This difference is shown with the highest average value of 1.3 mucus cells/1000 µm^2 in the freshwater plant group, compared to 1.1 for the freshwater marine group, followed along with 0.7 for both saltwater groups. External cells/1000 um^2 showed no significant difference between the groups ($p = 0.36$) (Figure 22, blue bars).

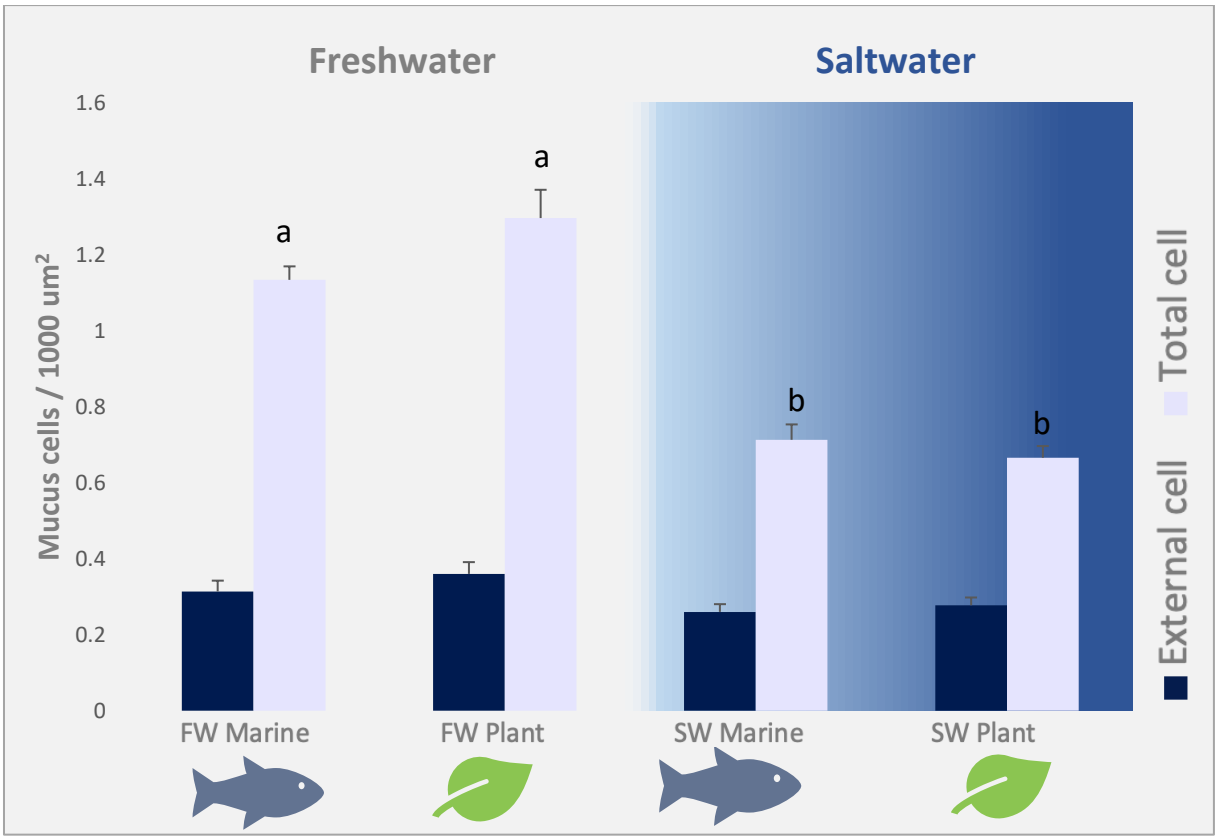


Figure 22: Grouped bar chart showing mucus cells/1000 um^2 (y-axis) of Atlantic salmon smolts, for the four following groups (x-axis) separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant): "FW Marine", "FW Plant", "SW Marine" and "SW Plant". The purple bars indicate total mucus cells/1000 um^2, whereas the blue bars indicate the external mucus cells/1000 um^2. Significant differences between groups within the same variable (external or total cells) are indicated with different letters above each bar. Each average value for the given bar is given inside the bar, and the standard error (SE) is shown by the error bars. The significance level is $p < 0.05$.

8.3.3. MUCUS CELLS AND EPIDERMIS AREA

The salmon sampled in freshwater had significantly higher amount of mucus cells/epidermis than salmon sampled in saltwater (FW Marin = 65.1 and FW Plant = 77.6, $p < 0.0001$) (Figure 24). Salmon fed plant diets had significantly larger epidermis area (SW Plant 72887µm^2 and FW Plant 65336.6 µm^2) than the marine diet (SW Marine = 49738.6 µm^2 and FW Marine = 54777 µm^2, $p = 0.0021$) (Figure 23). The number of mucus cells increased as the epidermis area increased and vice versa (Figure 23).

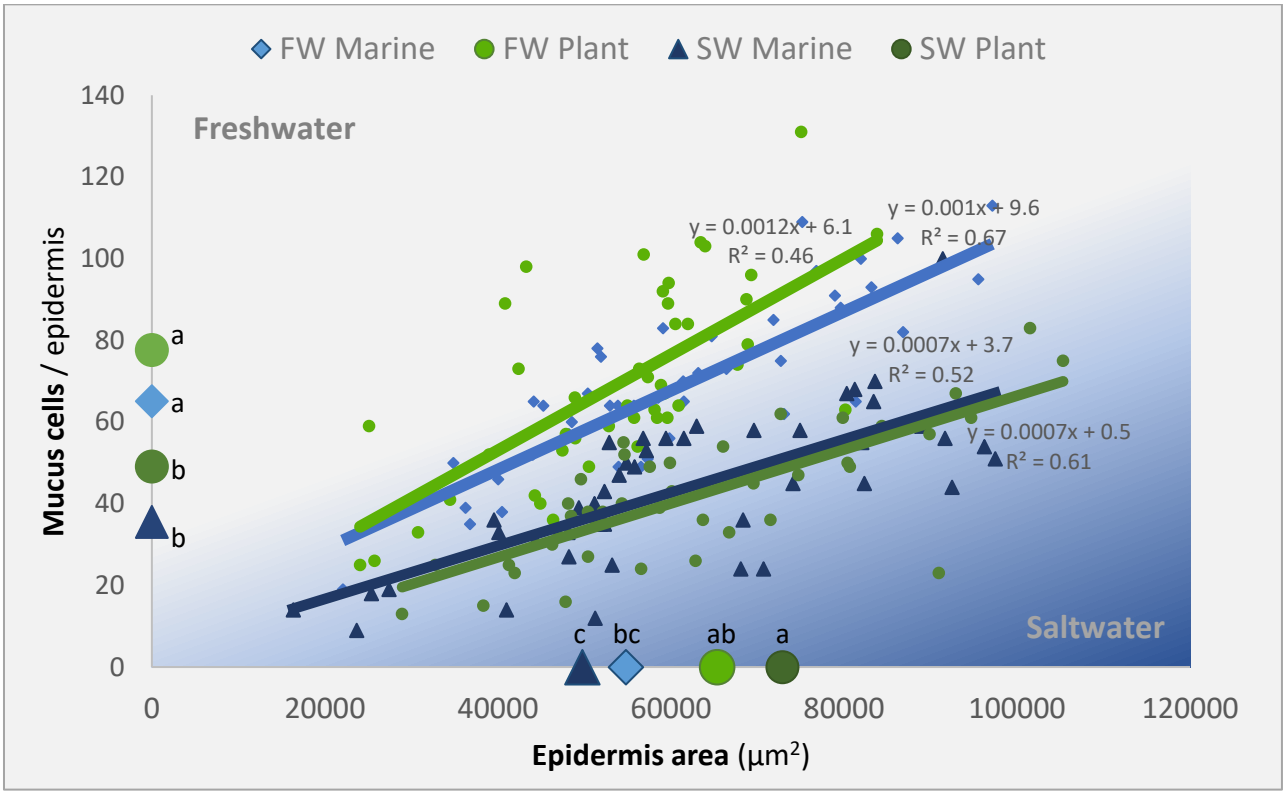


Figure 23: Scatterplot showing the amount of mucus cells/epidermis and epidermis area (µm^2) of Atlantic salmon smolts, separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant), resulting in the four following groups (indicators separated by colours): "FW Marine" (light blue), "FW Plant" (green), "SW Marine" (dark blue) and "SW Plant" (dark green). The results from each group are displayed with one linear regression trendline, specified with its equation and R^2. The blue background displays the region where the majority of the saltwater indicators are observed. In addition to the individual results, the average values for each group (when corrected for fish weight) are plotted on its representative axis (y-axis = average amount of mucus cells/epidermis when corrected for weight, x-axis = average epidermis area when corrected for weight). Significant differences between groups are indicated with different letters above/beside each average indicator on each axis. The significance level is $p < 0.05$.

8.3.4. Distribution of internal and external mucus cells

The relative amount of external (blue bars) and internal (purple bars) mucus cells changed significantly after saltwater transfer ($p = 0.0186$) (Figure 24). FW Marine had the lowest relative number of external cells (26%), followed by FW Plant (27%), SW Marine (36%) and SW Plant (40%). Consequently, the relative distribution of external mucus cells increased after seawater transfer.

The diets had no significant impact on cell distribution ($p = 0.6687$). However, the marine diet had the numerically lowest amount of external mucus cells in both environments, i.e. 1%-unit less than the plant diet in freshwater and 4%-units less than the plant diet in saltwater.

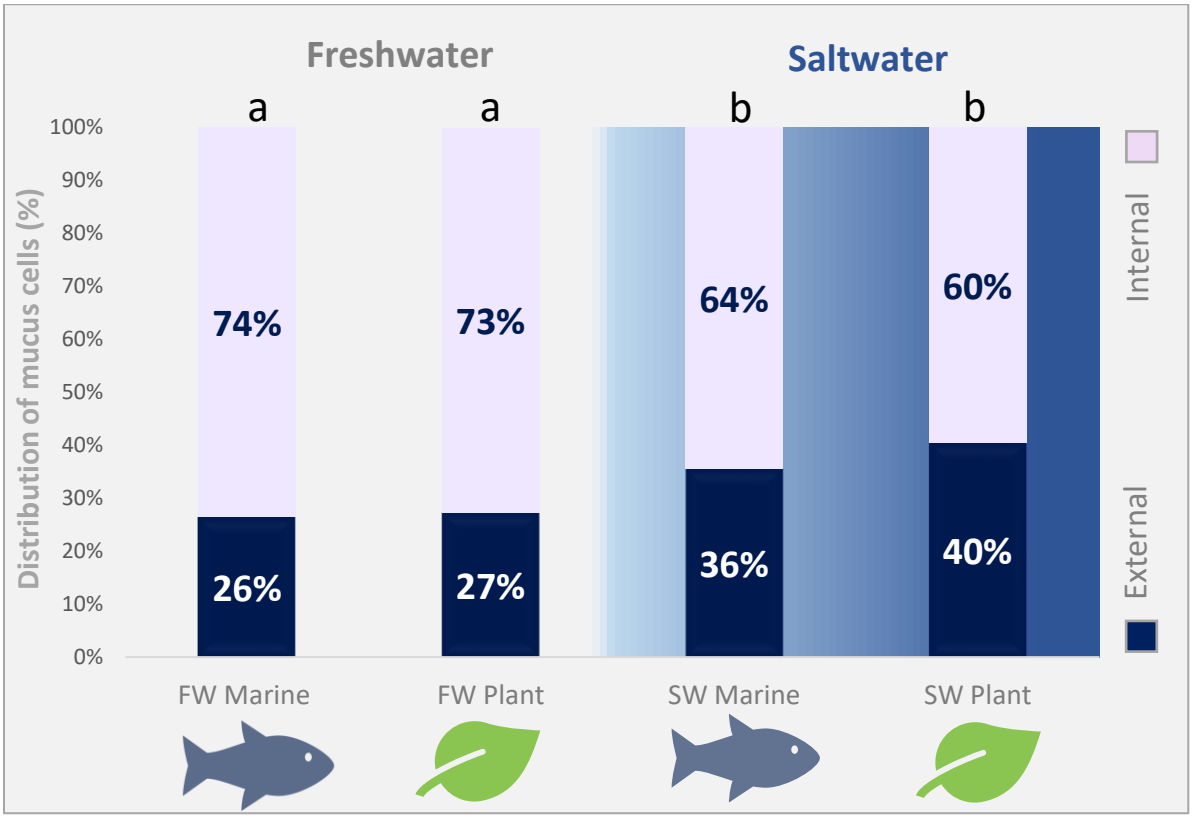


Figure 24: 100% stacked column chart, showing the average relative amount (%, y-axis) of internal (purple bar) and external (blue bar) mucus cells on each epidermis layer of Atlantic salmon smolts, for the four following groups (x-axis) separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant): "FW Marine", "FW Plant", "SW Marine" and "SW Plant". Significant differences between groups within the same variable (external or total cells) are indicated with different letters above each bar. Each average percentage for the given bar is given inside the bar. The significance level is $p < 0.05$.

8.3.5. EPIDERMIS THICKNESS

The epidermis layer of the SW Plant group was significantly thicker than the three other groups, both for end- ($p = 0.0315$) and mid-thickness ($p < 0.0001$) (Figure 25). The end-thickness (blue bars) was on average 31 µm, which represents 29 µm for both freshwater groups, followed by 31 µm for SW Marine and an increased thickness with 36 µm for SW Plant (Figure 26). The mid-thickness (purple bars) was on average 56 µm (corrected for fish weight differences), 25 µm longer than the average end-thickness. The plant groups showed the largest increase in mid-thickness, rising by 14.4 µm after saltwater transfer. In contrast, the marine groups decreased by 1.1 µm after seawater transfer.

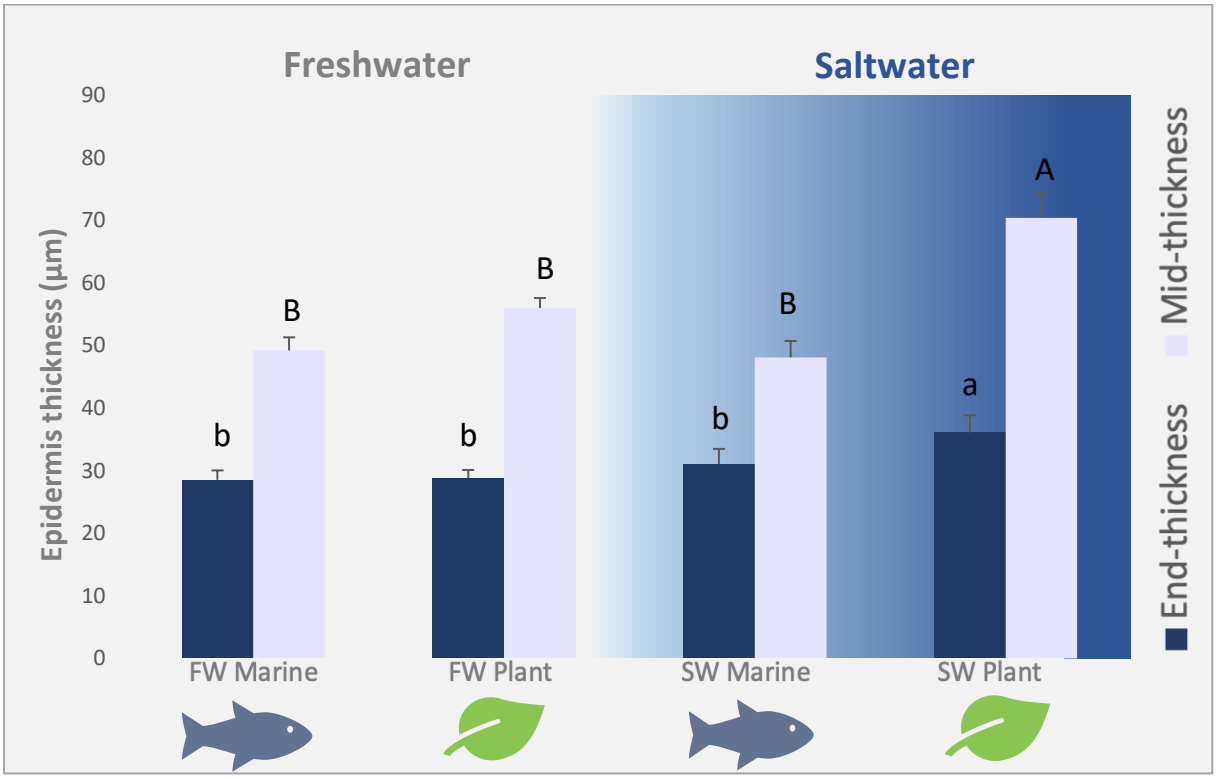


Figure 25: Grouped bar chart showing the average epidermis thickness (y-axis, µm) of Atlantic salmon smolts, for the four following groups (x-axis) separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant): "FW Marine", "FW Plant", "SW Marine" and "SW Plant". The grey bars indicate the average thickness of the 20% outermost part of the epidermis, whereas the red bars indicate the average thickness of the 20% mid-part of the epidermis. Significant differences between groups within the same variable (end-thickness or mid-thickness) are indicated with different letters above each bar. Each average value for the given bar is given inside the bar, and the standard error (SE) is shown by the error bars. The significance level is $p < 0.05$.

8.4. MICRO-LEVEL: GENE EXPRESSION

To study the changes in gene expression of the epidermis, qPCR analysis was performed on three stress related genes *iNOS*, *Hsp70* and *MMP9* and the reference gene EF1a. Here, the relative expression values were average to the determined "control" group, which was SW Marine. Figure 26 presents all results relative to this group. qPCR results showed that only iNOS was significantly differentially expressed and exhibited lower expression in the SW Plant group. The following results were found for each of the genes analysed:

- *iNOS* ranged between 1.48 (FW Plant) and -1.27 (SW Plant) log2 fold-change.
  The expression of the SW Plant group was significantly lowest (p = 0.0007).
- *Hsp70* ranged between 0.41 (FW Plant) and -0.43 (SW Plant) log2 fold-change.
  No significant differences were found between the groups (p = 0.1774).
- *MMP9* ranged between 0.10 (FW Marine) and -0.16 (FW Plant) log2 fold-change.
  No significant differences were found between the groups (p = 0.6864).

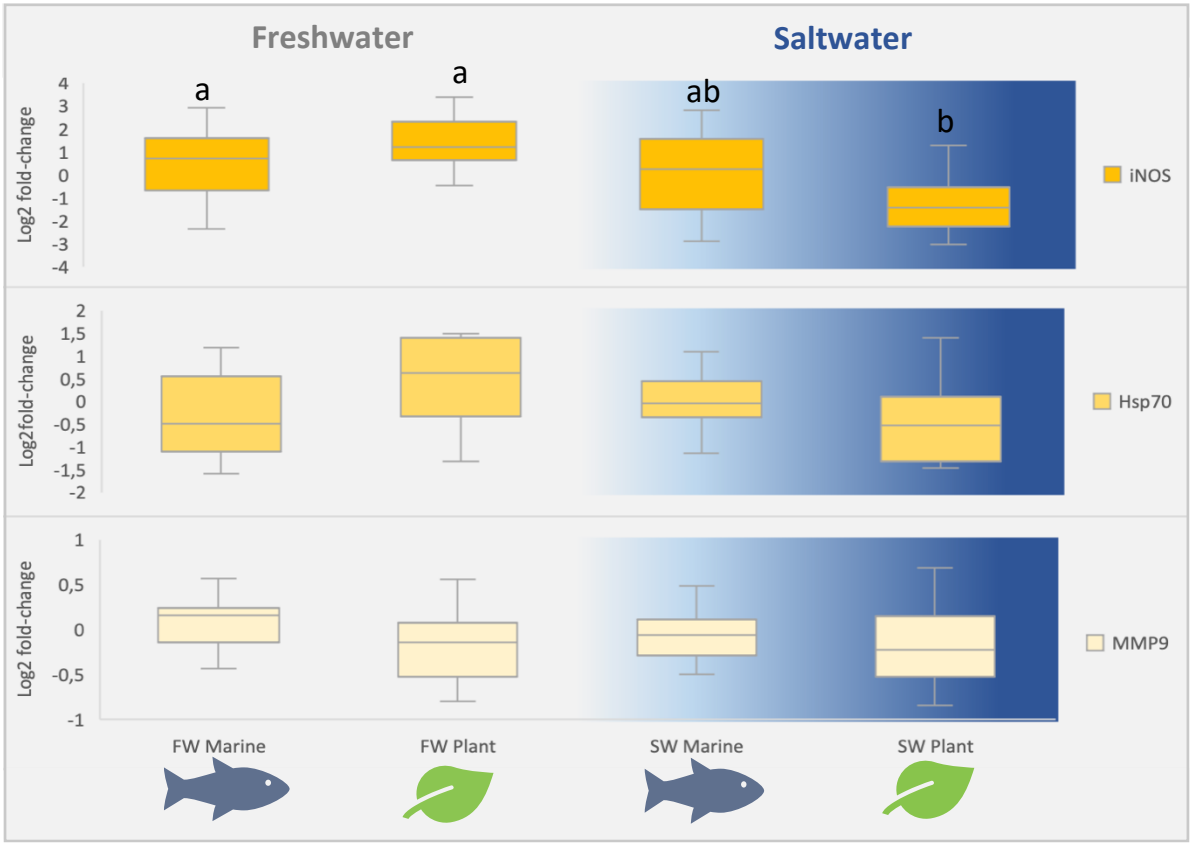


Figure 26: Box plot showing the average gene expression (y-axis) of Atlantic salmon smolts, for the four following groups (x-axis) separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant): "FW Marine", "FW Plant", "SW Marine" and "SW Plant". The genes tested were iNOS (yellow), Hsp70 (brown) and MMP9 (light yellow). Significant differences between groups within the same variable (gene expression) are indicated with different letters above each bar. Standard deviations (SD) are shown. The significance level is $p < 0.05$.

8.5. CORRELATIONS BETWEEN CONTINUOUS VARIABLES

The overall differences between groups (Figure 27) were caused by salinity (vertical comparison) rather than diet (horizontal comparison). Also, many of the histology variables were highly correlated, especially in saltwater. Additionally, *iNOS* showed an average correlation of 0.38 against mucus cells/1000 µm^2.

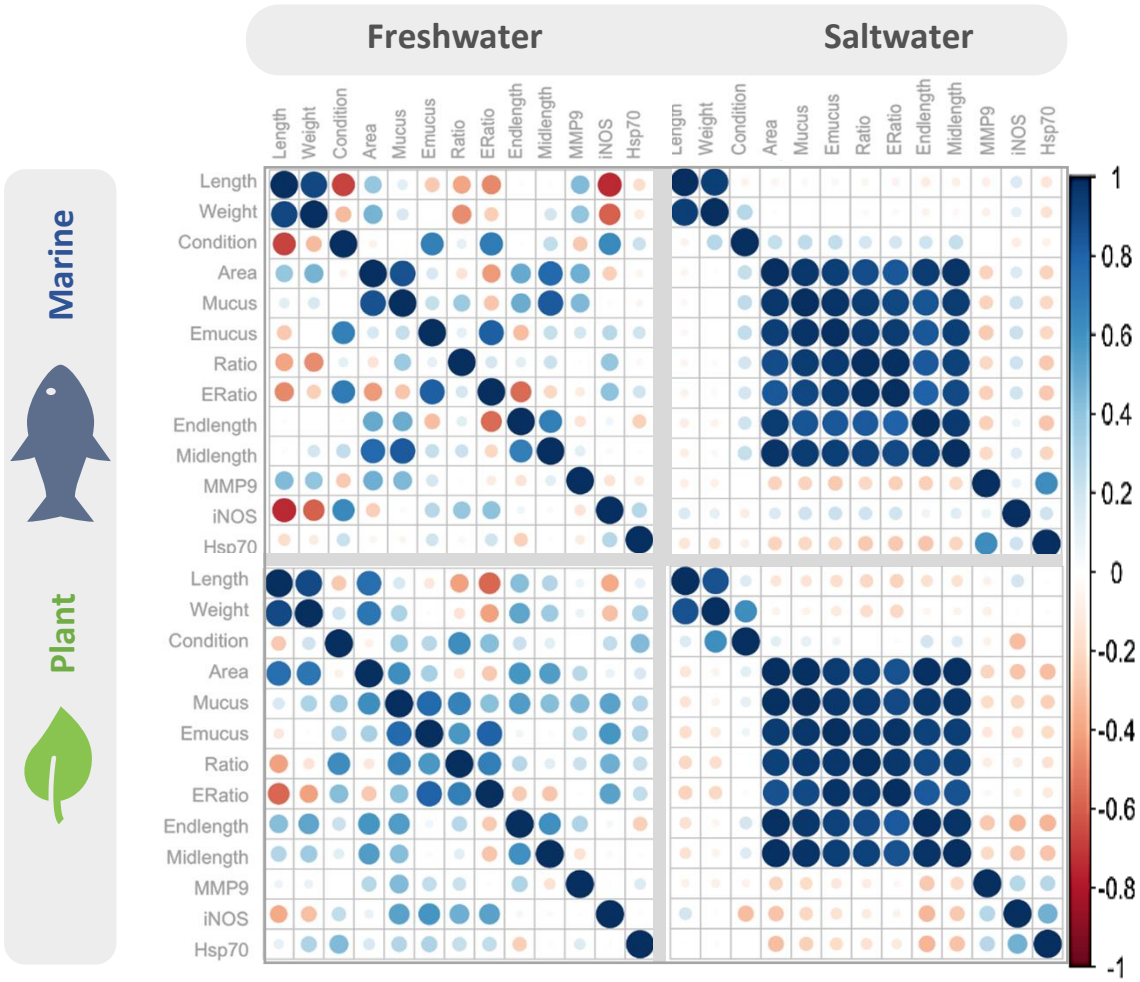


Figure 27: Correlation plot illustrating the correlation between continuous variables (x and y-axis) of Atlantic salmon smolts, including size measurements (length, weight, condition factor), histological skin measurements (epidermis area, mucus cells/epidermis, external mucus cells/epidermis, mucus ratio, external mucus ratio, epidermis end-thickness, epidermis mid-thickness) and epidermal skin gene regulations for three genes (MMP9, iNOS, Hsp70). The plot is divided into the four following groups separated by salinity (FW = Freshwater, SW = Saltwater) and diet (Marine, Plant): "FW Marine" (top, left), "FW Plant" (bottom, left), "SW Marine" (top, right) and "SW Plant" (bottom, right). The circle area and colour (right bar) determine the correlation size. The correlation increases with the circle's size and darkness, meaning that a strong correlation (close to 1 or -1) is illustrated by a large circle with dark colour and vice versa for a small/light/non-existent circle (close to 0). Additionally, the direction of the correlation is determined by the colour. Blue colour means positive and red means negative correlated.

9. DISCUSSION

9.1. TEST FEEDS

The advantage of testing a complete marine (except carbohydrate binders) against a complete plant diet is that they display two extremes. Using these strong contrasts makes detecting differences (in skin or growth) easier than only testing specific nutrients or raw materials. However, the downside is that it is not clear which specific nutrients are causing different phenotypes (skin morphology). Also, the results would likely be different if novel feed ingredients with different chemical compositions were used. Therefore, it is uncertain which results that can be extrapolated for new feed ingredients included in the future feeds.

Even though this trial showed that the diets influence the skin to a certain extent, the feed must be evaluated in context with other essential organs and body measurements, as intestines and hepatosomatic index. Additionally, genotype by environmental interaction (GxE interaction) could influence the results since the fish might be genetically predisposed to one of the feeds (Morais et al., 2012). To summarize, a holistic approach must be used to evaluate the diets used in this study.

9.2. FISH WEIGHT

The salmon fed a marine diet was larger by weight compared to salmon fed a plant diet. The weight differences could be caused by several factors, including the feeds' digestibilities. Plant diets contain more antinutrient factors than marine diets, which could decrease digestibility and thus affect the weight. For instance, phytates could reduce the bioavailability of certain essential trace elements, including Zn (Berntssen et al., 2010; Denstadli et al., 2007). Also, marine ingredients may have better palatability than plant ingredients, which will lead to increased feed intake (Espe et al., 2006). Additionally, only the marine feed contained EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). DHA and EPA requirements are life-stage dependent, and a recently published study reported that Atlantic salmon parr could increase growth performance by including these fatty acids in the diet (Qian et al., 2020). However, the diets of wild juveniles do not contain much marine ingredients, and plant ingredients could be expected to meet nutritional requirements. A study performed on parr showed that the fish could reach the same size by replacing marine protein with plant protein (lupin and pea protein) (Carter & Hauler, 2000). Nevertheless, diets based on plant ingredients require a higher supplementation of micronutrients (Vera et al., 2020) and the fish in this experiment were given the same micronutrient supplementation. The present study analysed salmon prior to and just after seawater transfer. It would be interesting to study the long-term effect of freshwater diet during the early stage for further studies, although former studies have indicated that freshwater diets do not necessarily have a long-term effect (Sissener et al., 2020).

9.2.1. WEIGHT VARIANCE

All groups except SW Marine had a quite similar and low weight variance. Interestingly, the exception in the SW Marine was relatively extreme since several individuals were in the lower quantile of the overall weight results (lower than most plant observations). Thus, it might be expected that many fish in this group had not eaten after seawater transfer. Therefore, the results that depended on fish weight were corrected for this parameter (weight), which is crucial since the experiment is a feed trial.

Perhaps, the greater variance of the SW Marine could be caused to hierarchy. High weight variances could signify sub-optimal social hierarchies caused by competition or feed deprivation (Cutts et al., 1998; Noble et al., 2007). Thus, the high weight variance found in the marine group after seawater transfer would not be optimal in commercial farming. Nevertheless, dorsal fin damage, which is a sign of aggression, was not observed in this experiment and is neither impacted by diet composition (Trovaag, 2019). However, aggression can occur without causing fin damages and the weight variance could also be affected by the sex composition in each group. Males could have a greater growth rate than females (Thorland et al., 2020). Perhaps, the marine group had a higher number of males than the plant group after saltwater transfer and therefore showed both increased growth *and* greater weight variation in saltwater. Also, the genetic weight potential could vary between individuals in general (Sonesson et al., 2013; Tsai et al., 2015).

Another hypothesis regarding the high weight variance in SW Marine is that this group were less adapted to the commercial feed (containing most plant ingredients) when transferred to saltwater than SW Plant. Hence, the growth performance after saltwater transfer could be influenced by the feed history in freshwater (Bendiksen et al., 2003), and it would be interesting to examine whether a slighter dietary shift could decrease the high weight variance.

9.3. CONDITION FACTOR

The condition factor was not influenced by diet but decreased in saltwater. A reduced condition factor after the saltwater transfer is expected (Noble et al., 2018), likely since the fish uses more energy on saline adaptation than body deposition (Alne et al., 2011; Handeland et al., 2003). Therefore, it seems difficult to influence the condition factor through diet composition during smoltification. In this experiment, all average condition factors for all the four groups were above 1. This could indicate that none of the dietary groups were malnourished (Noble et al., 2018).

9.4. SCALE LOSS

The majority of salmon had no or low scale loss (92%), but a trend could be seen with more scale loss in saltwater. This trend would also be the expected outcome, as smoltification can lead to loose scales (Takle et al., 2015). Therefore, the smoltification process is likely to impact scale loss more than the diet. The nonsignificant effect of diet on scale loss is also previously shown (Trovaag, 2019).

It is uncertain whether the results in saltwater can be extrapolated to the industry. Usually, the fish would be transported to seawater cages rather than tanks. A previous study in the same project (EX-Spot) showed that transfer to seawater cages led to significantly more scale loss than fish transferred to tanks (with saltwater) on land (Trovaag, 2019). However, this was an experiment performed on on-growing fish. Together, these results can strengthen the evidence on scale loss in different environments and diets, showing that environment but not diet could lead to more scale loss.

9.4.1. OVERALL CONDITION AND DEVELOPMENT OF THE FISH

According to FISHWELL´s morphological operational welfare (OWI) scoring (Noble et al., 2018), no wounds or severe damages were found on the skin. Also, no major morphological issues (such as deformities) were found, indicating acceptable welfare for both diets. However, one fish with greenish skin colour was observed in saltwater, perhaps evaluated as a precocious mature fish (Noble et al., 2018; Saunders et al., 1982). Therefore, the results (also for histology and gene expression) might be influenced by a rapid smoltification (0+) since this could lead to excessive stress and other results than for 1+ (Alne et al., 2011). According to the resource allocation theory (Cutts et al., 2002), rapid smoltification would mean more energy used for saltwater adaptation than skin maintenance. A major impact on rapid smoltification is shown on the salmon heart, causing cardiac deformities in later production stages (Frisk et al., 2020). Consequently, it would be interesting to examine if the salmon skin would also respond differently to a less intensive production method in freshwater.

9.5. HISTOLOGY

9.5.1. MUCOSAL CELLS

The skin of freshwater fish had more mucus cells than the saltwater fish, both in relation to cells/epidermis layer and cells/epidermis area. Likewise, previous studies have shown that the number of mucous cells decreases after smoltification (Karlsen et al., 2018), and it could even be an indicator of smoltification. This decrease occurs due to a change in the immune activity, possibly due to a reduction in prolactin-hormone levels (O'Byrne-Ring et al., 2003; Shephard, 1994). It is previously speculated that the observed modulation of the Atlantic salmon skin during the first post-smolt stage could increase susceptibility to infectious agents and risk of diseases during this first post-smolt period.

The skin of fish in the plant diet group had (slightly) more mucosal cells in both environments. Overall, fish mucus provides both physical and antimicrobial protection may act as a stress response system (Kulczykowska, 2019), and (Bates et al., 2006) respond to environmental challenges (Fernández-Alacid et al., 2018). Previous studies have also found that nutrition influences mucus composition (Djordjevic et al., 2021; Jensen, 2015) and that the diet in early life stages could impact the number of mucous cells later (Ruyter et al., 2016). For instance, Zinc is an essential component of the skin barrier. A study on postsmolts found that greater omega-3 levels in the diet improved zinc absorption (Berge et al., 2019). Therefore, the marine diet could have an advantage by its high levels of EPA and DHA. Also, feed with high levels of certain amino acids, especially threonine, could benefit mucus production and strengthen the barrier (Sissener et al., 2020). However, the amino acid composition is not known for the diets in this study.

It is challenging to consider which amounts of mucus cells that is the most preferable. Skin morphology could impact enzyme secretion and pathogen specificity. Fast (2002) found that species with more mucous cells and thicker epidermis, like the rainbow trout (*Oncorhynchus mykiss*), had greater lysozyme activity. High lysozyme levels are advantageous due to their bactericidal impact (Fast et al., 2002). Based on this, the greater number of mucous cells in the plant diet could be advantageous, potentially causing higher lysozyme levels. Conversely, Atlantic salmon with a low density of mucous cells are more resistant to salmon louse

(*Lepeophtheirus salmonis*) (Holm et al., 2015), signifying that a balanced ratio of mucous cells could be necessary for the protection of this demanding parasite. The advantage of a balanced mucus cell amount is also shown for intestinal mucous cells, being an essential barrier against pathogens, whereas too many occur after intestinal irritation (Kousoulaki et al., 2015a).

9.5.2. Internal and external mucus cells

The relative number of apically placed mucus cells increased after seawater transfer. Even though the diet showed no significant impact on these cells, the marine diet had the lowest relative amount in both environments.

Migration of mucous cells to the outermost part (apical membrane) of the epidermis could indicate stress (Sveen et al., 2020). Consequently, the marine groups showed the most promising results regarding cell migration and stress. Otherwise, the amount of apically mucous cells could be related to water temperature (Quiniou et al., 1998). However, all groups in this experiment were exposed to the same water temperature.

9.5.3. Epidermis area

The number of cells increased as the epidermis area increased. Accordingly, the plant diet (containing more cells) had a larger epidermis area than the marine diet. The large epidermis area for the plant diet relates to the thicker epidermis since both measurements evaluate the epidermal size. In contrast to epidermis thickness, limited research is previously performed on the epidermis area. Consequently, there is no clear explanation of the plant diet's larger epidermis area. However, it could indirectly be studied through comparing studies on epidermis thickness.

9.5.4. EPIDERMIS THICKNESS

The SW Plant group showed the most considerable epidermal thickness, both for mid- and end-thickness. The mid-thickness was, on average thicker than the end-thickness. Interestingly, the marine groups appeared on approximately the same epidermal thickness (slight decrease after seawater transfer), whereas the plant groups' thickness increased significantly after seawater transfer.

The epidermal thickness can vary according to sexual maturation, season (Rydevik, 1988), species (Fast et al., 2002), infection (Appleby et al., 1997), diet (Kato et al., 2014) and salinity. The latter could be why the thickness in this experiment was larger in saltwater. However, there is no clear evidence on why the diet did appear differently. High zinc levels could lead to a thicker epidermis and more mucous cells (Berge et al., 2019; Ruyter et al., 2016). In contrast, one former study showed that diets with variable EPA and DHA levels did *not* directly impact epidermal thickness (Cheng et al., 2018) but rather the skin's barrier function. In this experiment, EPA and DHA were only found in the marine diet, meaning that these components did likely not impact epidermal thickness results. However, as mentioned above, greater omega-3 levels could improve zinc absorption. In this term, the marine diet is perhaps expected to show the most considerable epidermal thickness, but this experiment showed the opposite outcome.

9.6. GENE EXPRESSION OF THE EPIDERMIS

9.6.1. OVERALL EVALUATION

The skin of fish is influenced by its environment, including stress (Karlsen et al., 2021; Sveen et al., 2016). Also, transfer to saltwater is challenging to the skin barrier functions and epidermal integrity (Karlsen et al., 2018). Here, the effect of diets on stress markers was evaluated. Out of the tested stress-related genes, *iNOS* showed reduced expression in the SW Plant group. However, the mechanisms behind and which components causing this outcome will need further studies. Expression of *HSP70* and *MMP90* showed non-significant alteration by diet and effect of seawater transfer. It is difficult to indicate any trend based on the selected stress marker genes. The variation in numerical values between samples is high in all groups, leading to a slight increase or decrease of means of the relative expression levels. The variation could be related to the method, i.e. scraping of the epidermis, or variability in tissue status of the sampled fish. The epidermis of fish is very dynamic with cellular turnover and migration of keratocyte cells to maintain epidermal integrity. Thus, the expression of the marker genes could vary between individuals. The reason for this observation might be that smoltification can lead to remodulation of the skin with high activity, which also could influence stress-genes. Similar, the skin is still adjusting to the new environmental conditions after saltwater transfer. However, during the first saltwater stage, the skin could prioritize other mechanisms than these genes (Johansson et al., 2016). Initiation of expression of these genes may also return to normal levels as the time between seawater transition and sampling may have been too long to include these genes as suitable stress marker genes.

9.6.2. INDUCIBLE NITRIX OXIDE SYNTHASE (*iNOS*)

Expression of *iNOS* showed downregulation in the plant group after saltwater transfer. Interestingly, the expression of iNOS was highest in the skin of the freshwater plant group. This could signify that the freshwater plant diet caused a different mobilisation of stress responses after saltwater transfer. *iNOS* controls baseline mucous production (Kousoulaki et al., 2015b) and Atlantic salmon smolts fed a diet supplemented with the microalgae *Schizochytrium* showed an increased gene activity of *iNOS* (Kousoulaki et al., 2015b). Thus, it is possible to impact the regulation of *iNOS* through feed ingredients, found both previously and in this experiment (where plant ingredients caused downregulation of *iNOS* and more mucosal cells). However, it is uncertain *why* the plant diet did respond differently. Also, *iNOS* could be upregulated by increased salinity for *postsmolts* (Takle et al., 2015). A regulatory change by increased salinity could also be indicated in this study for smolts in the plant group.

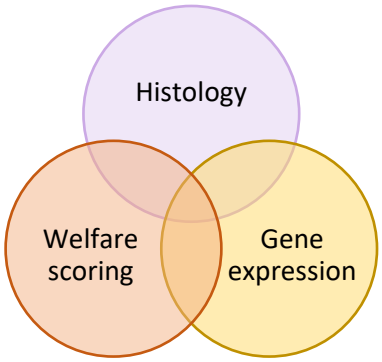
9.6.3. HEAT SHOCK PROTEIN 70 (*HSP70*)

Hsp70 are chaperones that are included in protein folding processes (Mayer & Bukau, 2005). Heat shock proteins could increase when exposed to several stressors (Roberts et al., 2010), and functional feeds could change the amount of heat shock proteins (Jensen, L. B. et al., 2015a). Also, HSPs in Atlantic salmon (Jensen, L. et al., 2015) and trout (Burkhardt-Holm et al., 1998) can be upregulated when exposed to high water temperatures (16C). Additionally, they increase in amount when exposed to parasitic sea lice (Provan et al., 2013). However, no significant difference of expression was detected in this study.

9.6.4. MATRIX METALLOPEPTIDASE 9 (*MMP9*)

MMP9 showed no differences between the groups, neither in terms of diet or salinity. Thus, the gene was not sensitive to dietary nor environmental changes in this experiment. Matrix metallopeptidases (MMPs) are enzymes that break down the extracellular matrix (ECM). Thus, they work as degrading proteases, essential in the first stages of wound repair and inflammation (Dahle et al., 2015). Matrix metallopeptidase 9 (*MMP9*) encodes for a protein that is central in the regeneration of scales in Zebrafish (*Danio rerio*) (Sahlmann et al., 2013; Takle et al., 2015). Due to MMP9s central function in wound repair, the gene would perhaps show different outcomes *if* the fish had wounds or more significant skin damages (scale loss, bleedings).

9.7. CORRELATIONS SHOW SALINITY TO BE THE STRONGEST DRIVER



The correlations plots showed more similar outcomes when comparing salinity rather than diet, indicating that the salinity had the most significant impact on the skin of the salmon smolts. Moreover, low correlations were found between the sampling levels (histology vs gene expression and fish size) – especially in saltwater. This could emphasize the importance of analysing parameters on several levels when evaluating salmon skin in saltwater. However, the correlation between the *iNOS* gene and mucus cells is an interesting interaction between levels in both this and previous findings (Kousoulaki et al., 2015b). Otherwise, the interactions occur on a highly complex molecular level, and the outcomes are thus difficult to explain, but the findings are interesting for further research. Moreover, it would be preferred to include welfare scoring in the correlation plot, but this could only be performed if the scoring method would be continuous (performed by analysing tools) rather than categorical numeric (performed by human evaluation).

9.8. GENERAL ASPECTS

9.8.1. KNOWLEDGE EVALUATION

Despite the knowledge learned through literature; this thesis is written with the absence of background from veterinary/fish health management. However, this thesis is not focusing on pathology. Also, the methods and results are developed and evaluated by researchers with a high expertise within biological fields.

9.8.2. FURTHER RESEARCH

For further research, the following aspects could preferably be added as extra parameters to provide a broader understanding of dietary effects on *skin health* of Atlantic salmon smolts:

- Cell size (hypertrophy)
    - Some mucous cells were observed as smaller in freshwater than in saltwater. However, this subjective assumption is not validated.
- Dermis (thickness and gene expression)
    - Dermal measurements were eliminated in this study but would provide a broader understanding of salmon skin.
- Skin colour (RGB analysis)
    - Skin colour could be an indicator of stress (Noble et al., 2018). Hence, a colour index could be made.

10. CONCLUSION

- Atlantic salmon smolts fed a marine diet in freshwater had a higher weight in both fresh- and saltwater.
- The dietary effect on skin morphology showed that the fish fed a plant-based diet in freshwater had a larger epidermis area and thicker epidermis in saltwater than those fed a marine diet in freshwater.
- The stress marker genes suggest no or limited effect of diet on the epidermis.
- The seawater transition reduced the condition factor. The skin responded by decreasing the number of mucus cells, while the proportion of apically (relative to internal) mucus cells increased.
- Scale loss was not significantly affected by saltwater transition or dietary composition.

11. APPENDIX

11.1. APPENDIX 1: DIET COMPOSITION

Diet composition		
	Marine diet	Plant diet
Fish meal	61	
SPC		34.6
Wheat gluten		22
Corn gluten		10
Wheat	20.68	8.37
Fish oil	15.4	
Rapeseed oil		20.4
MgSO4 (500ppm extra)	0.2	0.2
K2CO3 (500ppm extra)	0.1	0.1
Vitamin premix	0.5	0.5
Monosodium phosphate	2.5	2.5
Astaxanthin	0.05	0.05
Yttrium oxide	0.01	0.01
Mineral premix	0.5	0.5
Extra: Water adjustment	-0.98	0.73
TOTAL extra	2.88	4.59

11.2. APPENDIX 2: FEED INGREDIENTS' ORIGIN

Feed ingredients' origin	
Fish meal	Norse-LT, Vedde AS, Langevåg, Norway
SPC	Imcosoy 62 Aqua, Imcopa, Araucarias, Brazil
Wheat gluten	Amytex 100, Tereos Syral, Aalst, Belgium
Corn gluten	Glutalys, Roquette, Lestrem, France
Wheat	Norgesmøllene AS, Bergen, Norway
Fiskeolje	NorSalmOil, Pelagia, Egersund, Norway
Rapeseed oil	Crude rapeseed oil, Emmelev, Otterup, Denmark
MgSO4	Magnesium sulfate, Delivered by Vilomix, Hønefoss, Norway
K2CO3	Kaliumkarbonat, Delivered by Vilomix, Hønefoss, Norway
Vitamin premix	Nofima Vitmainpremix, Vilomix, Hønefoss, Norway
MSP	Delivered by Vilomix, Hønefoss, Norway
Asta	Lucantin PINK 10% from BASF, delivered by Vilomix, Hønefoss, Norway
Yttrium oxide	VWR, Oslo, Norway
Mineral premix	Nofima Mineralpremix, Vilomix, Hønefoss, Norway

11.3 APPENDIX 3: PHOTOGRAPHING

Photographing > Tissue sampling > Tissue sampling

1. Catch	2. Place the fish	3. Photographing
		
Catch the anaesthetized fish by holding under its right side.	Place the fish inside the photo box, next to its representative ID-tag.	Photograph the fish and its ID-tag from the hole above the photo box.

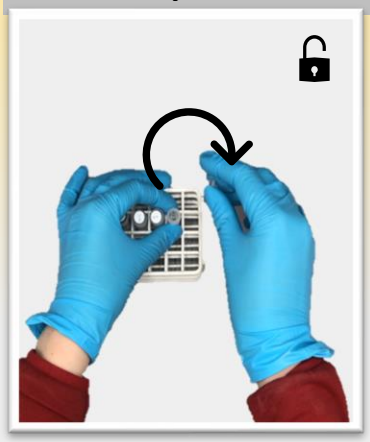
EQUIPMENT:

- Fish (anaesthetized)
- ID-Tags for each fish
- Gloves
- Camera
- Photo box (Camlink led photo studio)

11.4 APPENDIX 4: TISSUE SAMPLING FOR GENE EXPRESSION

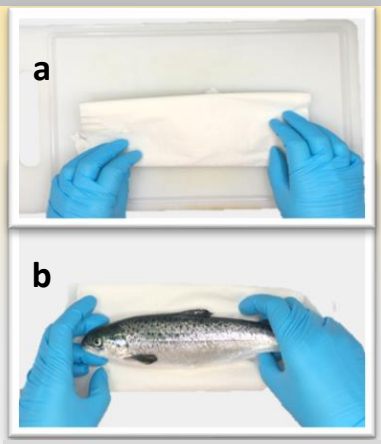
Photographing | **Tissue sampling** | Tissue sampling

4. Prepare tube



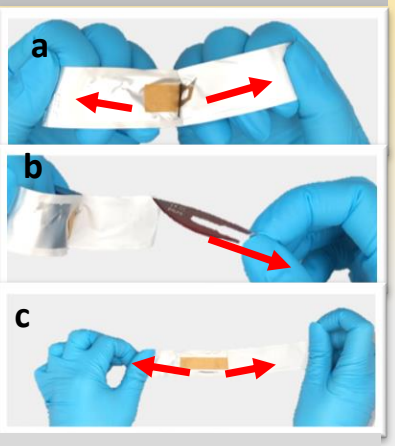
a) Find a tube with the correct ID for each fish.
b) Open the top of the tube.

5. Prepare substrate



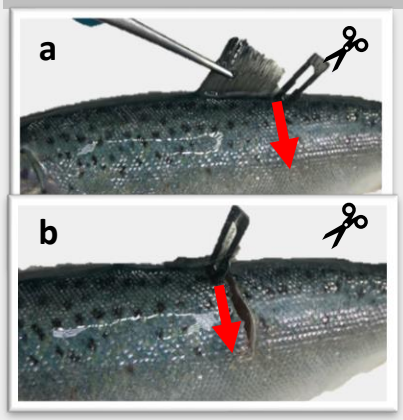
a) Put a paper on the substrate.
b) Place the fish on the paper.

6. Open scalpel



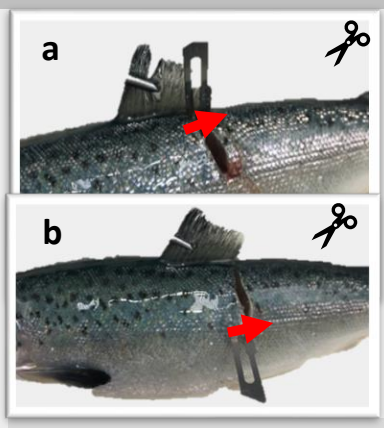
a) Open the folder halfway.
b) Drag the blade out.
c) Open the substrate and place it on the bench.

7. Ventral cut



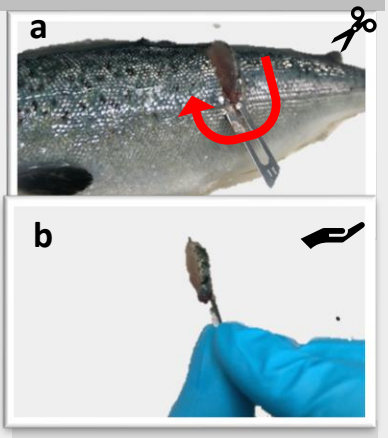
a) Use a scalpel to cut (~8 mm deep) from the caudal part of the dorsal fin, down to the lateral line.
b) Provide the same ventral cut, ~3 mm left to the latter.

8. Caudal cut



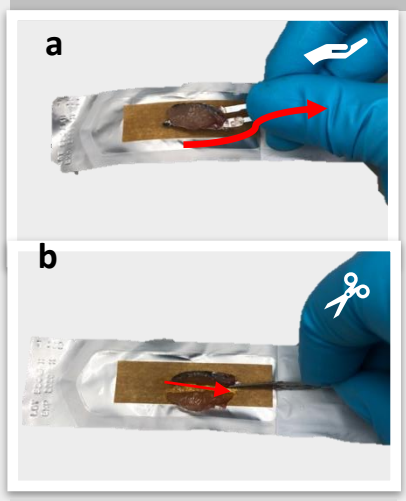
a) Cut (~8 mm deep) between the ventral cuts, along the dorsal fin.
b) Provide the same caudal cut, along the lateral line.

9. Horizontal cut



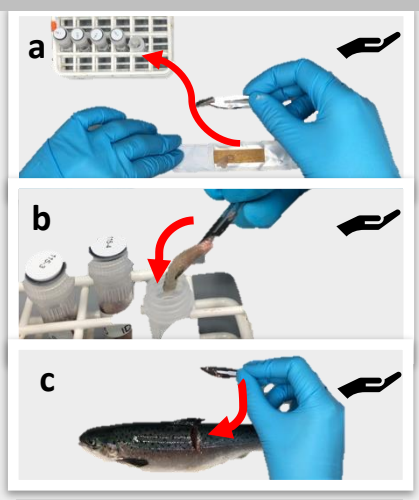
a) Cut underneath the vertical cuts.
b) Extract the biopsy out from the fish.

10. Prepare biopsy



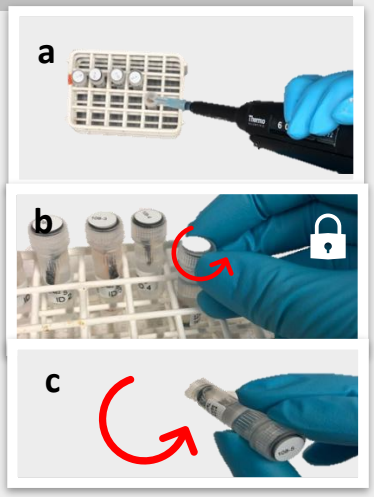
a) Place the tissue on the scalpel substrate.
b) Cut off additional muscle, so that the distance from scales to muscle has a width of ~3 mm.

11. Put biopsy into tube



a) Use the scalpel to lift the shortened bite up from the substrate.
b) Put the bite into the tube.
c) Place the scalpel into the recent cut.

12. Finish tube



a) Use a pipette to add additional RNAlater, until the tube is completely full.
b) Close the tube.
c) Turn the tube upside down once.

7. Additional steps

- Store the tubes in a refrigerator (-20°).
- Disinfect the tweezer with ethanol between each use.

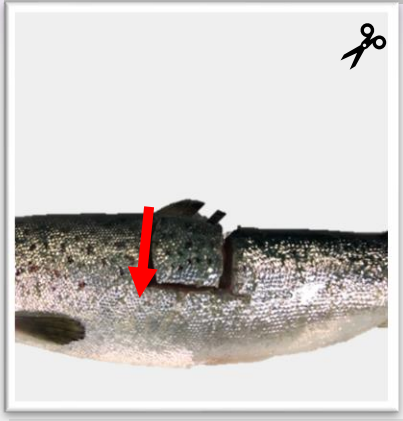
EQUIPMENT:

- Paper
- Scalpels
- Tubes with ID-tags
- RNAlater
- Ethyl alcohol
- Substrate
- Stand for RNAlater
- Pipette

11.5 APPENDIX 5: TISSUE SAMPLING FOR HISTOLOGY

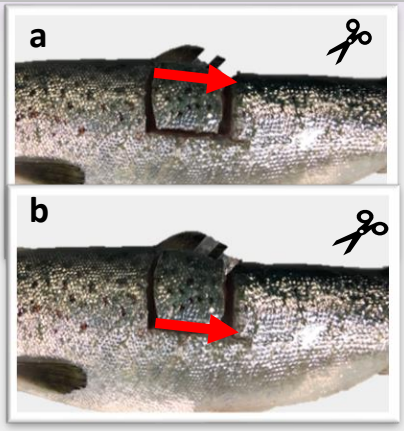
Photographing | Tissue sampling | Tissue sampling

13.Ventral cut



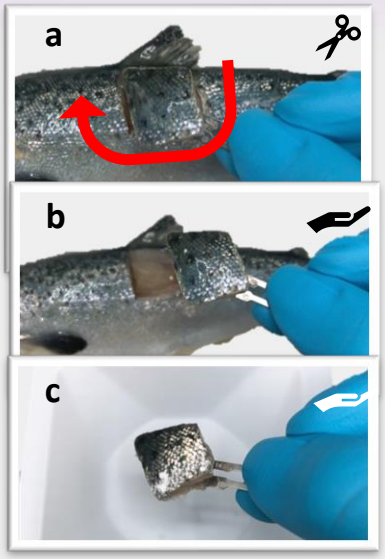
Use the scalpel to cut (8 mm deep) from the cranial part of the dorsal fin, down to the lateral line.

14. Caudal cut



a) Cut (~8 mm deep) between the ventral cuts, along the dorsal fin.
b) Provide the same caudal cut, along the lateral line.

15. Horizontal cut



a) Cut underneath the vertical cuts.
b) Extract the bite.
c) Lie the bite in a CellStor™ pot.

11. Additional steps

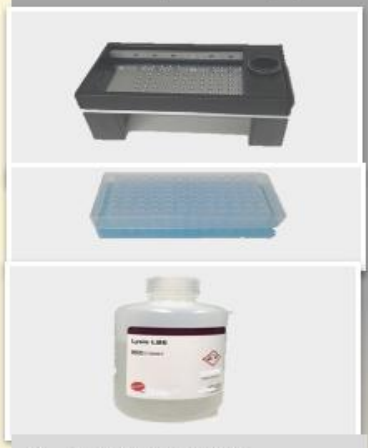
Store the bite in a tube with 10% buffered formalin. Send these to a lab where they are handled.

EQUIPMENT:

- Tube containing 10% buffered formalin
- CellStor™ pots, CellPath

11.6. APPENDIX 6: TISSUE PREPARATION FOR GENE EXPRESSION

1. Prepare equipment

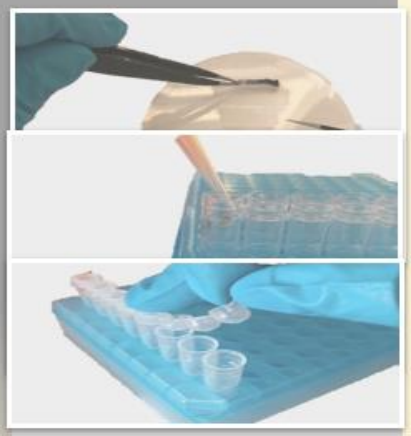


a) Add two magnetic densebeds into each tube on a Qiagen collection tube plate by using a distribution machine.
b) Use a pipette to add 400 ml lysis buffer into the test tubes.

2. Disinfect tweezer

a) Mark two 50 ml test tubes Ethanol 1 and Ethanol 2. Add 30 mL Ethanol into each test tube.
b) Dip the tweezers into the tube marked "Ethanol 1", followed by "Ethanol 2".
c) Use a paper to dry the tweezer. Make sure that no contamination occurs

3. Extract scales



a) Use a tweezer to extract 6-8 whole scales from the skin sample.
b) Add each scale into a 1 ml test tube. Make sure that all scales lie in the lysis buffer.
c) Add the lid to each tube.
d) Throw the scalpel.

4. Add proteinase K

Add 20 microliter Proteinase K (Beckman coulter) to each sample with Biosphere filter tips (2-100 microliter). Change the tip between each sample.

5. Shake samples

Put the plate in Fast Prep for 2 minutes and with maximum shaking 1800.

6. Centrifuge samples

Centrifuge the plate in a Beckman coulter centrifuge for two minutes. Make sure that the weight of the samples on each side are equal.

7. Heat samples

Furthermore, put the plate in Termaks heat cupboard for 60 minutes on 36,8 C°.

8. Freeze samples

Freeze the samples on -80 C°.

11.7. APPENDIX 7: RNA ANALYSIS

2100 expert_Eukaryote Total RNA Nano_DE72901876_2020-11-12_11-25-13.xad Page 1 of 18

Assay Class: Eukaryote Total RNA Nano
Data Path: C:\...Eukaryote Total RNA Nano_DE72901876_2020-11-12_11-25-13.xad

Created: 12.11.2020 11:25:13
Modified: 12.11.2020 12:20:53

Electrophoresis File Run Summary

Instrument Information:
Instrument Name: Bioanalyzer Firmware: C.01.069
Serial#: DE72901876 Type: G2939A

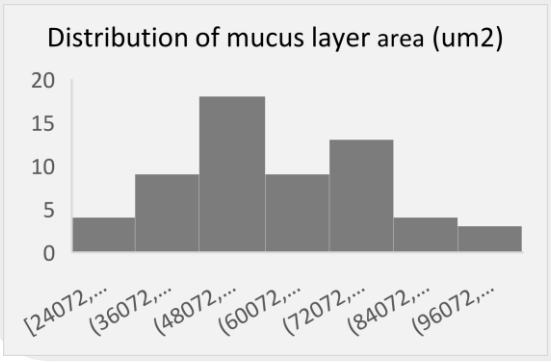
Assay Information:
Assay Origin Path: C:\Program Files (x86)\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Eukaryote Total RNA Nano Series II.xsy
Assay Class: Eukaryote Total RNA Nano
Version: 2.6
Assay Comments: Total RNA Analysis ng sensitivity (Eukaryote)

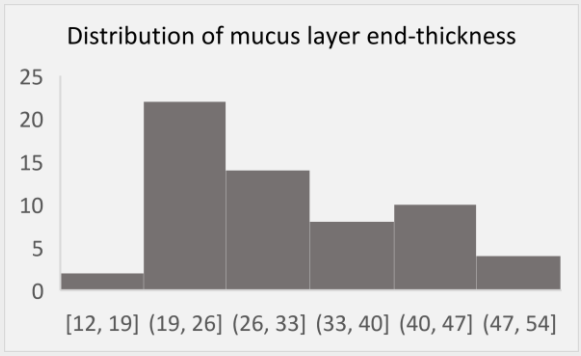
Chip Information:
Chip Lot #:
Reagent Kit Lot #:
Chip Comments:

2100 Expert (B.02.09.SI725)

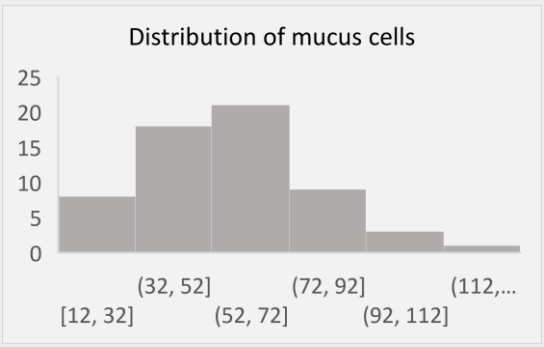
Printed: 12.11.2020 12:22:02

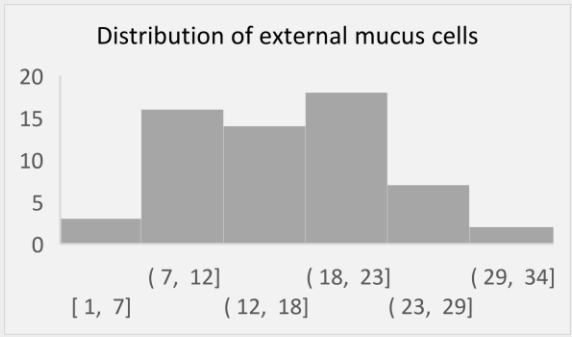
11.8. APPENDIX 8: NORMALLY DISTRIBUTED VARIABLES

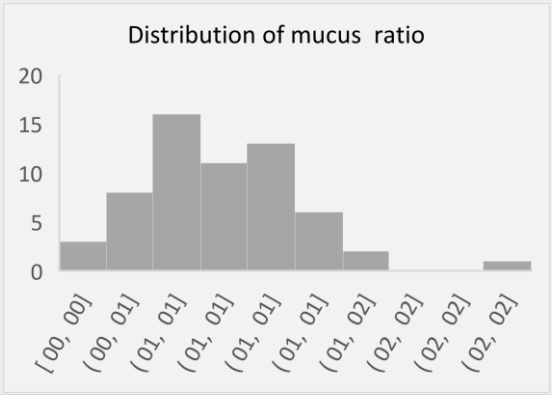


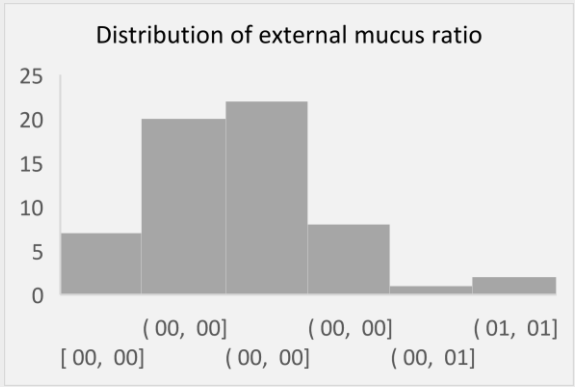


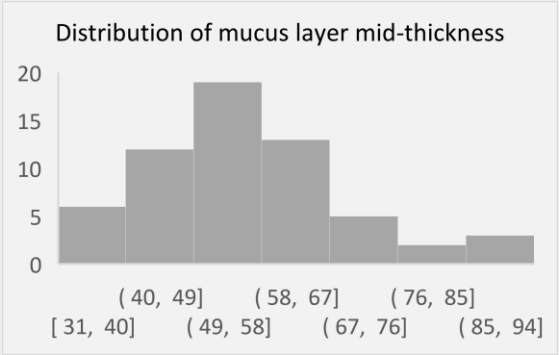
Non-Normal distributed variables











Normal distributed variables

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