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Impacts of climate-related temperature stress on Atlantic salmon (*Salmo salar* L.) gills

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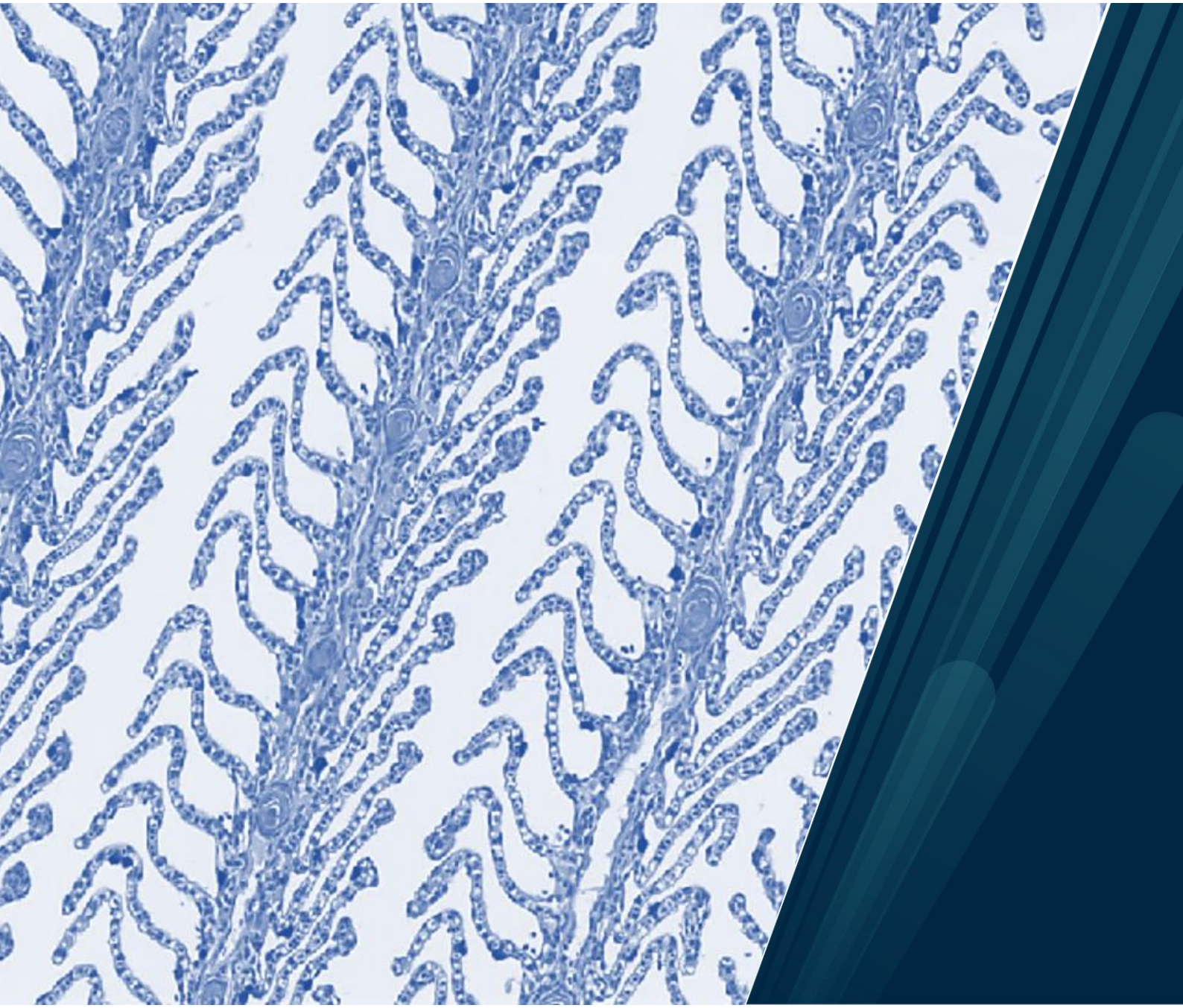

The Norwegian College of Fishery Science

Preface

This master's thesis was conducted at Nofima and symbolizes the end of my master's degree in Aquamedicine at the Norwegian College of Fishery Science, the Arctic University of Norway (UiT). The purpose of this study was to get a better understanding of how elevated and fluctuating temperatures, as an effect of climate change, will affect gill health of Atlantic salmon post-smolt. This study is a part of the Research Council of Norway project *Insight* – 194050.

I would like to express my deep gratitude to my supervisors Dr. Elisabeth Ytteborg and Dr. Carlo Lazado at Nofima. Thank you so much for the support and excellent guiding throughout my thesis, and for giving me the opportunity to be a part of the *Insight project*. It has been inspiring to have you as my supervisors.

A special thanks to Mira Hansen (Norwegian Veterinary Institute), for allowing me to participate in the processing of the histology sections, Ragnhild Stenberg Berg for the help and guidance with all the lab work and Gunhild Seljehaug Johansson for your help in understanding Aiforia®, as well as tips on how to set up nice tables and figures. Thank you Gerrit Timmerhaus for all the statistical analysis of the Aiforia® results – *R* is complicated stuff, and Jelena Kolarevic for helping me understand the growth performance.

Many thanks to my partner in crime Mina and the rest of the master's students at Nofima, for fun conversations and enjoyable coffee and lunch breaks. We made it!

Most of all, many thanks to Hanna, Mina, Nora, Solveig and Vilde for making these study years so memorable. It's going to be incredibly strange not being able to hang with you every day. Last but not least, a thanks to my family and boyfriend for believing in me and the support you have always shown me. Although it will be sad to move from Tromsø, a happy flatmate is waiting for me to move back to Trøndelag, where I will start my very first adult job.

Tromsø 15.mai.2023

Anne-Marja K. Jannok-Joma

Abstract

Constantly increasing water temperatures caused by climate change, can lead to major consequences for the welfare of farmed fish. The interactions between the expected changes and the fish physiology are still not understood, in specific how the changing environment may affect the fish's health, and how this may impact aquaculture production in general. The fish's gill health can be used as an indication of the fish's overall condition, and a greater understanding of this organ can help to influence decisions that salmon farmers make for operational procedures, e.g., by targeting particularly vulnerable environmental situations in combination with delousing and transportation. Identifying temperature thresholds and tolerance can lead to modified procedures for monitoring and handling of fish. In this thesis we have looked at how elevated and fluctuating temperatures affected gills in Atlantic salmon and compared it with fish from a control group at lower temperature. The hypothesis was that fish at elevated and fluctuating water temperatures may induce changes in gills that may further impact the fish overall robustness, compared to fish at constant lower temperature. Histological changes and gene transcription in gills related to temperature stress may lead to increased understanding of this topic. The results were consistent with the assumption, showing that elevated and fluctuating temperatures have an effect on gill health in Atlantic salmon, which is previously linked to negative effects for the fish. Gills from fluctuating temperature regime generally had several morphological changes compared to gills from the high-temperature group, including the presence of fusion, clubbing and lifting. Changes in gene responses were generally not observed, however, important markers for oxidative stress as *Cu/zn sod* were down regulated and *Mn SOD* were up regulated. Genes were mostly down-regulated in the high and fluctuating groups, indicating a general down-regulation of genes. Overall, results show that fluctuating and high temperature have significant impact on gill integrity in farmed Atlantic salmon, and that the changes caused by the different temperatures differs. This should be taken into consideration when planning future farming under climate change.

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1. Introduction

Aquaculture is key to meet the world's growing demand for food (FAO, 2022). The world's population is increasing at a rapid pace, and is estimated to reach 9.7 billion by 2050 (Gu et al., 2021). With the increasing population, the demand for food also increases. Aquaculture is one of the fastest-growing food production sectors in the world and is responsible for more than 50% of global seafood production. It includes animals, plants and microorganisms harvested through fisheries and aquaculture activities, whether marine or inland (FAO, 2022). In 2020, global aquaculture production reached a record of 87.5 million tons of aquatic animals, with grass carp dominating in global inland aquaculture, and Atlantic salmon (*Salmo salar* L.) in marine aquaculture (FAO, 2022).

Food and Agriculture Organization of the United Nations (FAO) has estimated that the total aquaculture production will reach 106 million tons in 2030 (figure 1), an overall growth of 22% compared to 2020 numbers (FAO, 2022). This increase in production is necessary to meet the growing consumer demand. However, the world`s climate has been experiencing rapid change over the past years, and climate change threatens global food production, including seafood. Climate change results in rising ocean temperatures, leading to unprecedented changes in global marine ecosystems, sea level rise, and longer and more frequent marine heatwaves (Oliver et al., 2018). How these changes will impact the production of Atlantic salmon in Norway is still debated. Large knowledge gaps exists when it comes to both the impact of climate change along the cost of Norway, and how the fish will respond to the coming changes (Falconer et al., 2022).

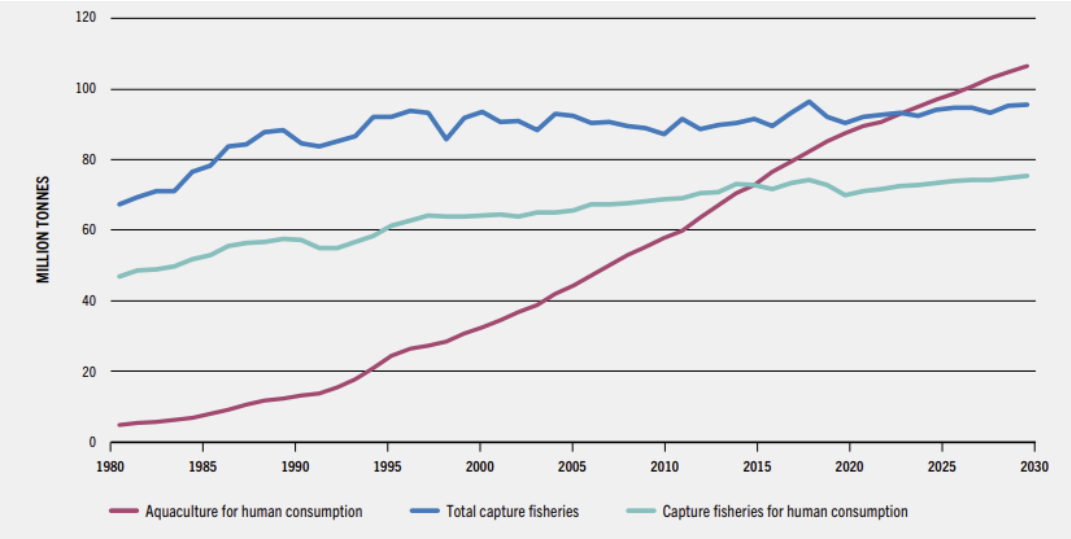

Figure 1. FAOs estimations of the global aquaculture production and total capture fisheries from 1980-2030. Retrieved from FAO, 2022.

1.1 Norwegian salmon farming

Norway is the world's largest producer of farmed Atlantic salmon, and salmon farming takes place along the entire coastline (FAO, 2018). The aquaculture industry in Norway started in the early 1960s by the brothers Vik being inspired by Danish freshwater farming of rainbow trout (*Oncorhynchus mykiss* W.) to start their own farming. They found that rainbow trout could gradually be acclimated to seawater, and through experiments with several different salmonid fish species through the 1960-70s, the first farmed salmon was slaughtered in 1971 (Aasen, 2011). This was the start of the subsequent development of farming of Atlantic salmon.

Atlantic salmon is among the most iconic and economically important fish species in the world, and Norwegian fjords have been shown to provide good conditions for farmed fish, because of their good water flow conditions and oxygen-rich water (NOU 2019:18, 2019). Since the production technique was pioneered in the 1960s, salmon production in Norway has increased steadily from 600 tons in 1974 to nearly 1.544.000 tons today (Fiskeridirektoratet, 2023; Olaussen, 2018). In conventional salmon farming, production starts in a hatchery, where roe is fertilized and hatched. The fish are kept in the hatchery systems until they undergo smoltification. The smoltification includes increased salinity tolerance, increased metabolism, downstream migratory, silvering, darkened fin margins and olfactory imprinting to make the fish ready for life in seawater (Björnsson et al., 2011). Sea water-adapted smolt are then transferred to sea-based cages, where they remain until harvest, around 15 months, depending on location and desired harvest size (Hansen, 2019). Compared to natural processes, salmon in nature will spend between 1 - 4 years in the sea before migrating back to river and steams for spawning (Wennevik & Hansen, 2020). In conventional farming, the post-smolt stage is believed to be the most sensitive and critical phase of salmon production for survival in the sea, with increased mortality and disease outbreaks, weakened immune system, reduced growth and reduced appetite (Iversen et al., 2005; Noble et al., 2018).

For many, the history of the Norwegian salmon aquaculture industry seems a success. But, as the aquaculture industry experienced explosive growth beyond the 1980s, the industry faced major challenges associated with disease problems, and at that time it was mainly bacterial diseases that caused problems (Mortensen, 1998; Sundell, 2023). Many of the diseases had high infection pressure, and in facilities with millions of individuals, the spread was fast, and mortality was high. In the 1980s, there were few solutions other than antibiotics, which led to

issues on resistance. The development caused concerns about whether the diseases would be a limiting factor in the industry, and work was done to find measures to limit antibiotic use, but also outbreaks and fish loss. Vaccine development, increasing knowledge of fish's immune system, genetics, environmental conditions and focus on fish health, as well as strict routine control, have led to focus on the prevention of disease (Sommerset et al., 2023). Nevertheless, mortalities are still increasing, and salmon lice and various types of virus diseases are still a widespread problem (Sommerset et al., 2023). With climate change on top of these problems, research on fish health is essential for further production and growth of the industry.

1.2 Climate change and aquaculture

The ongoing climate changes will affect aquaculture and fish health, but how and in what extent is still unknown. However, one thing is clear, the sustainability of future aquaculture is at risk due to the effects of the ongoing climate change, and these impacts have already caused high mortalities at several salmon farms around the world. The potential effects of climate change on aquaculture production and its implications for the sector's sustainability have been reported (Falconer et al., 2022; Maulu et al., 2021). Main climate stressors were categorized as sea level rise and extreme water levels, storms, air or sea temperature, extreme temperatures and heat waves, ocean acidification, deoxygenation and changes in precipitation and runoff (Falconer et al., 2022). Various impacted elements of a changing climate on marine salmon farming have been discussed; health and welfare, stress, feed, growth, diseases and harmful algae and jellyfish blooms (Falconer et al., 2022; Foyle et al., 2020; Maulu et al., 2021).

1.2.1 Elevated sea temperatures

Salmon is a cold-water specie, and the preferred water temperature depends on the different life-stages. In the seawater-phase, the upper preferred water temperature is 16 °C, with an optimal temperature between 10-15 °C (Handeland et al., 2003; Stien et al., 2013). Temperatures above 17 °C have been shown to cause reduced appetite, growth performance and increased mortality. As salmon are ectotherms, the environmental temperature controls their body temperature, which in turn affects vital processes such as metabolic rate (Kieffer et al., 1998). The salmon has thus occupied habitats with temperatures that best suit its body

function. Salmon avoids areas with temperatures from 18 °C and higher (Noble et al., 2018), but temperatures of 17 °C in a combination of reduces oxygen level have been shown to lead to high mortality (Burke et al., 2020). This observation was made in Newfoundland during a heat wave in 2019. Research by Folkedal et al., (2012) showed that post-smolt exposed to repeated temperature fluctuations had a lower feed intake compared to fish that were not exposed to temperature fluctuations. It has also been shown that post-smolt exposed to 18 °C eat less compared with fish kept at lower temperatures (8 °C and 12 °C) (Kullgren et al., 2013). These studies support the suspicion that higher temperatures reduce growth by disrupting the fish's metabolic processes, but can also lead to mortality.

Water temperature is also related to dissolved oxygen concentration. The higher water temperature, the less dissolved oxygen is in the water and available to the fish (Folkedal et al., 2012; Noble et al., 2018). Increasing temperatures can therefore cause areas to become more oxygen-limited for environmental-sensitive species (Klinger et al., 2017). The global average surface temperature is estimated to increase by 2 °C by the year 2100 (IPCC, 2023), which has further led to theories that the sea potentially can be warmer down to 3000 meters deep (European Commission, 2007a). Temperature increase can have both direct and indirect consequences on aquatic organisms, such as physiological processes including increased metabolism rate, muscle functions and growth, but also changed migration patterns (Little et al., 2020; Schulte, 2015).

Study looked at how increased temperatures affects the biomass distribution in the sea cage, showed that when the temperature increased, there was intense crowding in the cages with high biomass (Oppedal et al., 2011). This probably comes from when the water temperature increases, less dissolved oxygen becomes available, and the fish will search for areas with higher oxygen levels. Colder water has more oxygen, and the fish will therefore swim deeper in the sea cage. In that way, the vertical space with optimal oxygen levels becomes limited in the sea cages (Solstorm et al., 2018) and lead to the oxygen in the cage being used up and the fish eventually suffocates (Burke et al., 2020). Determination of locations and suitable species for production is likely to be a more and more relevant topic in the face of the ongoing environmental changes.

1.2.2 Marine heat waves

Extreme water temperature changes, with prolonged periods of abnormally warm ocean temperatures, called marine heat waves (MHW), are increasingly reported (Frölicher et al., 2018). These heat waves are seen as a threat to marine ecosystems and their functioning, by leading to widespread mortality of marine species and adaptive reconfiguration of species' ranges. This will further have an economic impact on the seafood industry, by leading to a decline in aquaculture production in commercial fisheries (Smale et al., 2019; Xu et al., 2022), as a result of reduced availability of important fishery species (Hobday et al., 2016; Sen Gupta et al., 2020). It is shown that average MHW frequency and duration have increased by 34% and 17% between 1925-2016. With this information, it is widely believed that these changes are due to an increase in the average sea temperature, which suggests that a further increase can be expected in the future (Oliver et al., 2018).

Studies of Atlantic cod (*Gadus morhua* L.) in the northwestern Atlantic have documented reduced population growth, suboptimal growth conditions and increased mortality as a response to long-term ocean warming. A decrease in survival was recorded in late-stage cod larvae and juveniles, which is linked to increased summer temperatures (Pershing et al., 2015). This means that cod will move northwards and into deeper water. In this way, fisheries in various areas will disappear, but become available in other (Mills et al., 2013). However, as different species have different sensitivities to sea temperature, thermal tolerance, dispersal capacity and ability to utilize available resources, thus species that originally live in one area may move as a response to warmer conditions (Fossheim et al., 2015). Farmed aquatic animals do not have the same possibility to move away from unfavorable conditions as the wild species have, since they are kept in cages. Knowledge on how salmon respond to environmental changes and how we can better protect the fish from challenging conditions are needed.

1.3 Gills

An understanding of gill structures and their functions are needed to be able to understand how a changing environment can affect the gills. The fish gill is a multifunctional organ responsible for a number of interconnected physiological processes; aquatic gas exchange, osmotic and ionic regulation, acid-base regulation and excretion of nitrogenous waste, in order to maintain the fish's systemic homeostasis in a changing internal and external environmental condition (Evans et al., 2005; Ferguson, 2006). The gills, in addition to the skin and the gastrointestinal tract, have mucus covered epithelium and make up the surface and the physical barrier between the internal and external environment. For that reason, these organs will act as first line of defense against pathogens (Ángeles Esteban, 2012; Cabillon & Lazado, 2019).

1.3.1 Anatomy of the gills

Atlantic salmon have four gill arches on each side of the back part of the oral cavity, shielded by the operculum. Water is taken in through the mouth and passed through the gills under the operculum. Through this process, the gills absorb oxygen. The gill filaments, called primary lamellae (figure 2A), are equipped with numerous, thinner vertical plates, called secondary lamellae (figure 2B), where the actual gas exchange takes place (Amin et al., 1991; Kryvi & Poppe, 2016).

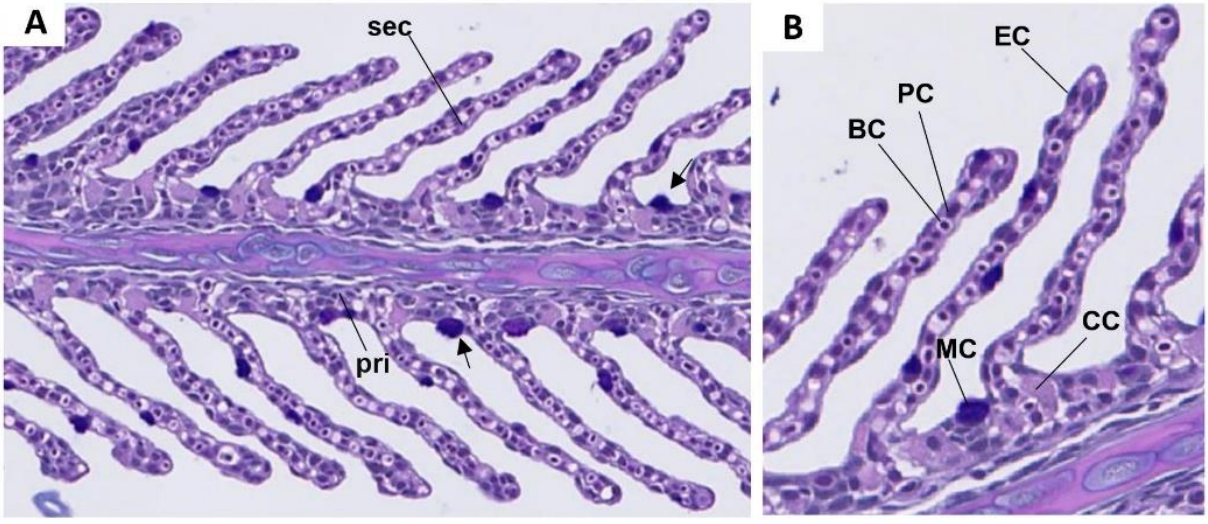

Figure 2. Primary and secondary lamella. (A) Primary lamella, pri, with equipped with numerous, thinner vertical plates, called secondary lamellae, sec. Arrow pointing on a mucous cell. (B) Close-up of secondary lamellae; EC, epithelial cell; PC, pillar cell; BC, blood cell/ erythrocyte; CC, chloride cell; MC, mucous cell. Gill samples stained with Alcian blue-Periodic acid-Schiff (AB-PAS).

The secondary lamellae consist of a thin single plate epithelium and pillar cells in the capillary wall. The pillar cells are specialized endothelial cells, which form the capillaries, and provide strength and structure. Fish use the counter-current system to absorb oxygen via the secondary lamellas by water and blood cells, erythrocytes, from the gill arteries, flowing in each direction over the capillaries. Erythrocytes with low oxygen concentration will thus absorb oxygen from oxygen-rich water through passive diffusion (Schmidt-Nielsen, 1997). How good the exchange efficiency is, depends on, among several factors, the diffusion distance between the secondary lamella and the water. This efficiency can be limited by various gill irritations such as gill disease, water quality and changes in the environment, as they cause the lamellar thickness to increase (Ferguson, 2006). In this way, oxygen becomes less accessible to the fish, leading to reduced oxygen uptake and complications with respiration.

The chloride cells are found at the basis of the secondary lamella. These cells are larger than the epithelial cells and have bright cytoplasm and a basal core. In the cell membrane of the chloride cells, active secretion of Na^+-ions (sodium ions) occurs. The number of chloride cells is generally higher in marine and saltwater-adapted fish than freshwater fish (Ferguson, 2006). In fresh water, the fish will constantly try to counteract the passive loss of ions, and primarily sodium and chloride. This is done by absorbing ions from nutrients that sodium and chloride are absorbed over the gills. However, unlike fish in fresh water, fish in seawater are in a constant state of ongoing dehydration and must counteract the passive gain of ions and loss of water. This property manages the fish by drinking seawater and absorbing salt and water across the gut, while excess sodium and chloride are excreted via the gills (McCormick et al., 2009).

Mucous cells are cells with epithelial origin that function to produce mucus. The mucus produced is important for both immunological and mechanical protection of the lamellae. Mucus is a viscous colloid that contains inorganic salts, lytic enzymes such as lysozymes, immunoglobulin and glycoproteins. These help protect the fish from pathogens such as fungi, bacteria and viruses (Uribe et al., 2011). Mucus production also helps with dehydration and physical and chemical damage. When the mucins, which are the major macromolecular components of mucus, comes into contact with water, they are converted into viscous gel (Ferguson, 2006). Gills that become irritated, as in the case of water quality change, pollution or pathogenic attacks, the mucous cells will be able to proliferate, and mucus production will increase (Foyle et al., 2020). Various research projects on gill health have reported that such

conditions lead to increased number, distribution and change in the size of the mucous cells, indicating the protective functions of the mucus layer and its associated mucin against gill irritations (Alipio et al., 2023).

1.3.2 Gill health

Stressed fish and/or fish with gill damage have shown a lower tolerance for infectious pathogens, after being exposed to multiple stressful conditions such as environmental factors, management operations or a combination of these (Boerlage et al., 2020; Mitchell & Rodger, 2011; Rodger et al., 2011). Fish with gill irritations or gill damage receive recognizable behavioral change and typical clinical signs such as coughing, gasping after air, excessive mucus production, and fish standing against the flow direction. In addition, flared opercula and gasping may indicate a reaction to adverse environmental conditions (Roberts & Smith, 2011).

«Complex gill disorder» (CGD) is a term used when multiple disease types are present in the gills, unlike one primary type. This term is widely used in the aquaculture industry and refers to nonspecific gill disease, in which histopathological findings in the gills suspect that there are several infectious, and non-infectious agents, associated with the disease changes (Boerlage et al., 2020). Currently there are seven distinguishable types that refer to infection by one principal causal agent or insult: (i) amoebic gill disease (AGD), (ii) parasitic gill disease, (iii) viral gill disease, (iv) bacterial gill disease, (v) zooplankton (cnidarian nematocyst)-associated gill disease, (vi) harmful algal gill disease and (vii) chemical/ toxin-associated gill disease (Boerlage et al., 2020). According to the Norwegian Veterinary Institute's fish health report for 2022 (Sommerset et al., 2023) complex gill disease is one of the biggest health challenges for farmed salmon, and is the second biggest cause of reduced welfare in the sea phase.

Gill epithelial health and environmental factors influence on how well the fish can compensate for morphological changes and recover from infections. Fish has been shown to remodel the gill structure in response to salinity, hypoxia or acidification, yet increased temperatures have been associated with increased gill changes that can be seen in histopathology (Foyle et al., 2020). Histological indicators of gill disease include epithelial lifting and edema, curved tips and hyperemia of the lamellae, increased mucus secretion, necrosis, aneurism and lamellar

fusion as a response to increased temperatures (Liu et al., 2015; Prakash et al., 1998; Takata et al., 2018). Rohu fingerlings (*Labeo rohita*) have been shown to have a remodeling capability in a changing climate, to be able to withstand changes in water temperature (Islam et al., 2020). Gill remodeling has also been seen in crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) (Sollid et al., 2005), where the remodeling has been shown to be caused by an induction of apoptosis and cell cycle arrest in the mass of cells filling up the space between adjacent lamellae, causing this interlamellar cell mass (ILCM) to shrink. This leads to a reduced respiratory surface area and thus a reduction of osmoregulatory costs due to lower water and ion fluxes. In order to understand what influences climate change have on gill physiology, more research is needed, acknowledging that gill health is a central component in fish health, but also in relation to food security (Foyle et al., 2020).

1.3.3 Methods for assessing gill health

There are various available techniques and methods used for assessing gill health. Macroscopic evaluation of the gills can tell something about the condition and damage of the gills. Gill status is checked by looking for changes such as mucus stains, bleeding, blood clots, colour and pale areas, and missing gill lamellae. Reasons for changes can be due to infections such as AGD shown in figure 3 (1A, 2A, 3A), algae, jellyfish, chemicals or mechanical damage, such as non-chemical delousing. Slightly reduced gill function is given a score of 1, a clearly changed gill with reduced gill function is given score of 2 (<50% of the tissues in the gill), and gills with major changes are given a score of 3 (>50% of the tissues in the gill). In research, the gill status must be checked by the same person throughout the trial period to avoid bias (Nilsson et al., 2022).

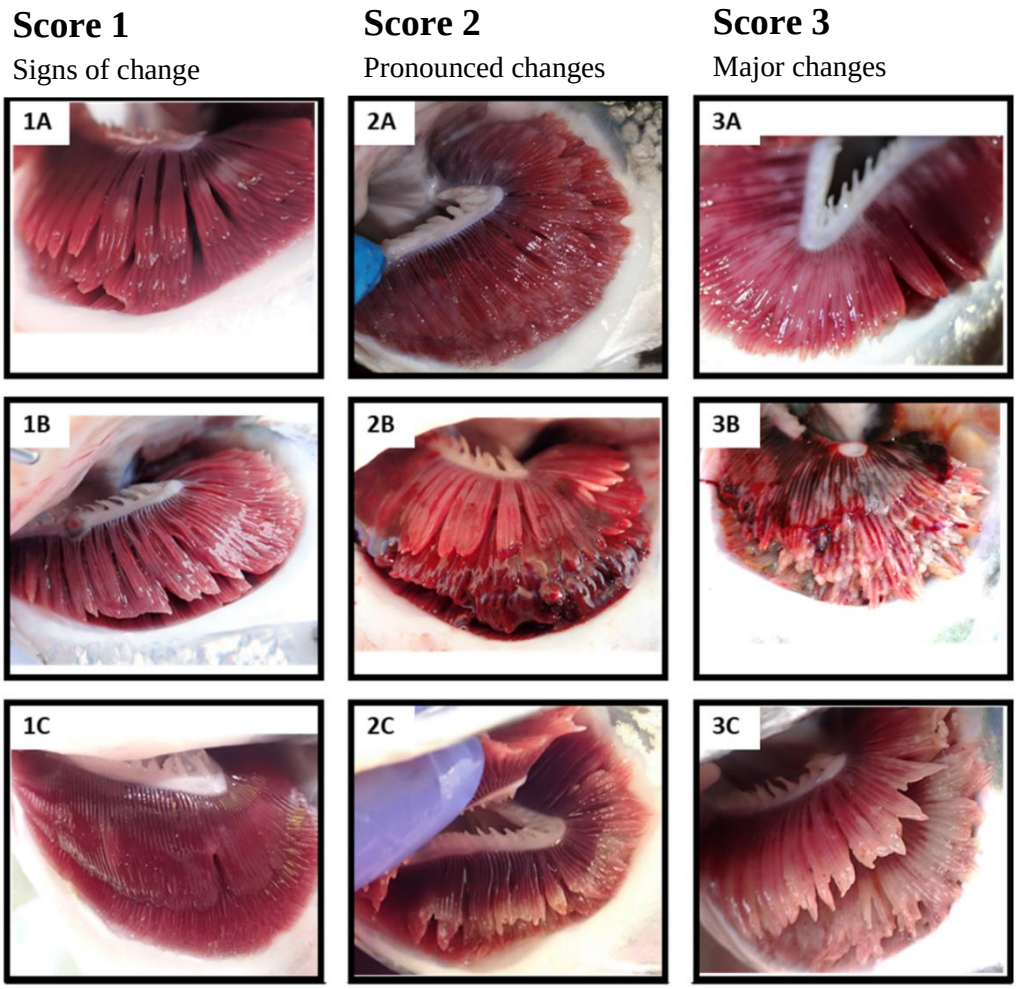

Figure 3. Macroscopic evaluation of gills in Atlantic salmon. Scoring scale goes from score 1- 3. Score 1 indicates there are signs of changes in the gills, score 2 shows that there are clear changes in the gills, and score 3 is given to gills with major changes. Illustration from the "Laksvel" protocol (Nilsson et al., 2022).

If disease is suspected or for other interests, such as research or to check gill status, samples of the gills can be sent to a laboratory for further evaluation. Laboratory-based methods include e.g. histopathological examinations and molecular methods. Through histology, the degree of tissue change can be assessed, and different colour methods provide the possibility of staining specific tissue components. Histological analysis can either be detected using manual scoring or through digital scoring platforms, such as Aiforia®. A disadvantage of manual histopathological scoring is that the interpretations are subjective. Nevertheless, a number of researchers have developed various evaluation systems that can be used as guidance for developing own scoring criteria (Lazado et al., 2022; Mitchell et al., 2012). The gill logarithm in Aiforia® has been taught to detect various tissue and cell components and makes it easier for

researchers to quickly get an overview of the gill status and are more objective. Still the results from digital histology have shown that the method needs optimalization when e.g. artefacts are present, as these changes are making it more difficult for samples to be correctly analyzed using artificial intelligence.

Molecular methods incudes e.g., qPCR analysis, DNA-microarrays and RNA sequencing. qPCR is a very specific and sensitive method of analysis with a function to detect desired genes in organisms and is a useful tool for assessing the immune status of the fish, by looking at whether the relative expression of genes is present or absent in the gills. Combining different approaches provide the opportunity to test specific responses, e.g., immune and stress responses, in one organ to see if e.g., a specific treatment affects the integrity of cellular structures, tissue quality and vital functions. Nevertheless, qPCR has a disadvantage which is hypothesis driven, that it only shows which genes are expressed at the time of sampling, and therefore only a selected number of genes can be investigated. Stress, caused by for example handling during sampling, can be expressed in the results, and help to influence the actual effects/treatment that one wants to look at. However, by sampling from all tanks at the same time, one tries to reduce this element of the analyze.

1.4 Aim of study

Rising water temperatures as an effect of the ongoing climate change has been shown to increase the risk of welfare and health issues in Atlantic salmon and is a central problem in today's aquaculture industry that urgently needs to be addressed.

This master study was a part of a bigger project conducted by Nofima (Insight, The Norwegian Research Council (NRC) grant agreement number 194050). The main aim of this study was to test the effect of suboptimal (17 °C) and fluctuating (12/17 °C) temperatures on gills of post-smolt Atlantic salmon and compare them with a control group (12 °C). Three sub-objectives were defined:

- Assess general fish appearance at different temperatures
- Evaluate histological sections of gills to search for temperature related effects by using a manual scoring system combined with digital histology using the Aiforia® platform
- Study expression of selected genes associated with stress in gills

The hypothesis was that fish in elevated and fluctuating water temperatures may induce changes in gills that may further impact the fish overall robustness, compared to fish at constant lower temperature, and that there are differences in how gills respond to constant high and fluctuating temperatures in regard to damages and recovery potential.

2. Materials and methods

2.1 Ethical statement

The described study was approved by the Norwegian Food Safety Authority (FOTS ID 29728) and conducted in accordance with the regulations controlling experiments and procedures for live animals in Norway.

2.2 Experimental fish

Atlantic salmon (*Salmo salar*) was hatched and smoltified by NRS Settefisk (Dåfjord, Ringvassøya, Tromsø, Norway), in freshwater flow-through system under constant daylight (LD 24:00). All fish were vaccinated with Alfa Ject 6-2, a 6-component injectable vaccine containing inactivated *Aeromonas salmonicida*, *Listonella anguillarum serotype O1*, *Listonella anguillarum serotype O2a*, *Vibrio salmonicida*, *Moritella viscosa* and *infectious pancreatic necrosis virus* (IPNV) (PHARMAQ, Norway).

Post-smolt with a starting weight of 97 g ± 16.3 g, were transferred to the Aquaculture Research Station (Havbruksstasjonen) in Kårvika (Tromsø, Norway), divided into six 500-liter tanks, with 100 individuals in each tank, and acclimatized to a flow-through system in the Fish Health Laboratory, for three weeks prior to the trial. The water quality was kept at an optimum level for post-smolt: salinity of 33ppt, dissolved O_2 was monitored and kept >90 % and water flow on 6 -7 L/min. The fish were continuously fed with Olympic 3mm (Skretting, Norway).

Fish were kept at three different temperature conditions for two months: optimal 12 °C, high 17 °C and fluctuating 12/17 °C conditions. Two rounds of fluctuating conditions were done, lasting three weeks each, with samples taken prior to temperature increase (from 12 °C to 17 °C), at the peak (17 °C) and after returning to 12 °C. The fish were sampled at the end of the wave to get most of the temperature effect, and a day after the fish returned to 12 °C. The water temperature in the fluctuating group increased from 12 °C to 17 °C with 0.5 °C per day, lasting 10 days. The fish were then exposed to high temperature (17 °C) for 5 days, before the temperature decreased with 0.5 °C per day down to 12 °C (lasting 10 days).

This was done to gain an understanding of how the fish in the fluctuating temperature (FT) handled two "heat waves" compared to one round, to see whether the fish in the high temperature (HT) and the FT were able to adapt to these water temperatures over time. This was to see whether the fish suffered more or less damage, and whether the histological observations showed that the gills were able to regenerate during the trial period or whether they had more histological changes. These observations were compared with the gills from fish in the control temperature (CT). An overview of the experiment is illustrated in figure 4.

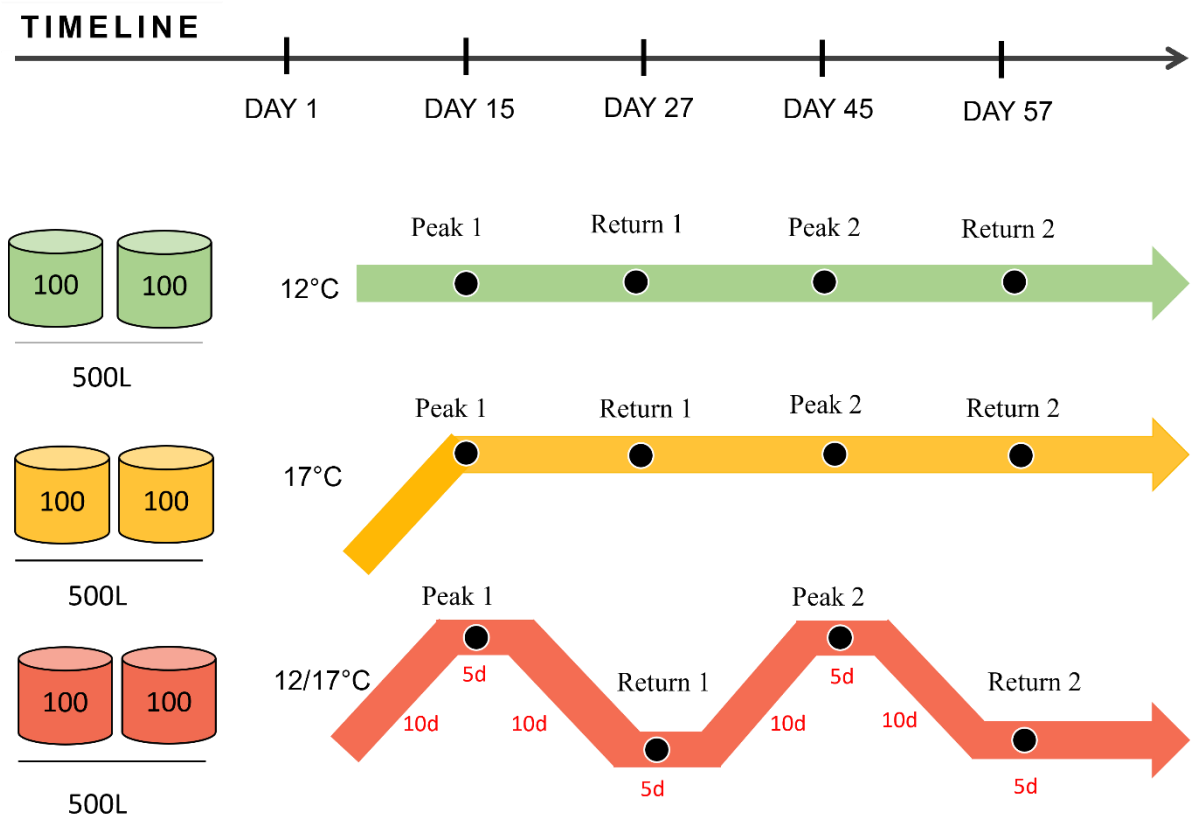

Figure 4. Experimental design of the fish trial showing the duplicated tanks and the temperature profile of the different tanks. Black dots illustrate different time points, and d = days.

2.3 Tissue sampling

Five fish from each tank were sampled at the different time points after being humanely euthanized by an overdose of anesthetic added to the water, as described in the protocol Benzoak vet (new name Optomease Vet, ACD Pharmaceuticals AS, Norway).

Welfare scores and fish data, including fish identity, length, weight, and scale-loss were scored and registered. Samples from the fish's mucus layer and plasma were collected. As this master's project is part of a larger *Insight project* (Norwegian Research Council, grant agreement NRC #194050), sampling was also conducted on organs that are not included in this thesis; blood, mucus, skin and muscle, olfactory, liver, spleen, head kidney, heart and intestine. As several organs, especially gills, are exposed to enzymatic degradation, it was important to keep the sampling time as short as possible. The samples included in this study are shown in figure 5.

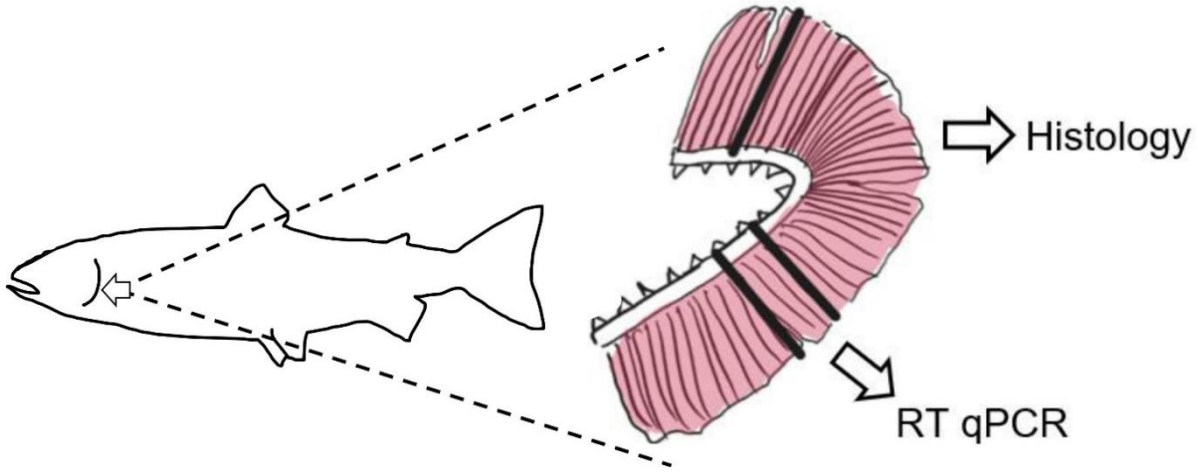

Figure 5. Sampling of gills for transcriptional analyses and histology. Samples for qPCR are approximately 0.5-1 cm wide.

Gill samples were taken from the second gill arch on the fish's left side for both histological and transcriptional analyses, so that the histology sample and real-time quantitative polymerase chain reaction (qPCR) sample are both from the same gill arch. The gill sample was excised using tweezers and scissors. The operculum and first gill arch were folded outwards so that the second gill arch became visible. To avoid damage to the filaments, the gill sample was carefully lifted using a tweezer and cut, not touching the tissue used for histology. The samples were taken as aseptically as possible by washing equipment with 70% ethanol (EtOH) (Merck, Rahway, NJ, USA) and changing scalpel blade between each fish.

Gill samples for histology were carefully placed in pre-labeled 20 ml pots containing 10% buffered formalin (Cellstor, CellPath, Newtown, UK) and stored at 4 °C until use. Sample for transcriptional analyses were placed in a 2.0 ml eppendorf tube (Screw cap micro tube 2 ml, Sarstedh AG, Germany) filled with 1 ml RNAlater solution (Invitrogen, Thermo Fisher Scientific, Wilmington, USA). Samples in RNAlater were kept at 4 °C overnight, before being stored at -80 °C until use.

It is important that the fixatives (formalin and RNA buffer) were within the tissue:solution ratio (minimum 1:10), so that the tissue samples were completely fixed.

2.4 Growth performance

Calculations of condition factor (K-factor), thermal growth coefficient (TGC) and specific growth rate (SGR) were calculated from weight and length of each fish, and are shown in Eq. 1, Eq. 2, and Eq. 3.

$$K - factor = \frac{W}{L^3} \cdot 100 \qquad \text{Eq.1}$$

W and L is respectively weight (gram) and length (cm) from the individual fish.

$$TGC = \frac{W2^{\frac{1}{3}} - W1^{\frac{1}{3}}}{T \cdot \Delta t} \cdot 1000 \qquad \text{Eq.2}$$

T is temperature (°C) and Δt is the time in days between the samplings (W1 and W2).

$$SGC = \frac{\ln Wt - \ln W0}{t - t0} \cdot 100 \qquad \text{Eq.3}$$

Wt is the average weight at time t, and W0 is the initial weigh at time t0.

2.5 Real-Time qPCR

qPCR was used to detect transcriptional differences in the gills using a selected set of genes. In this study, stress genes as *hsp70, hsp90, Cu/zn sod, Mn SOD, GPx,* pro-inflammatory *il1β,* apoptosis gene *casp3a*, pain gene *trpv1* as well as *claudin 10e* and *claudin 28a, collagen 1a* and the reference genes *Ef1α* (elongation factor), *β-actin* and *18S* was included. A schematic overview of the qPCR method is presented in figure 6.

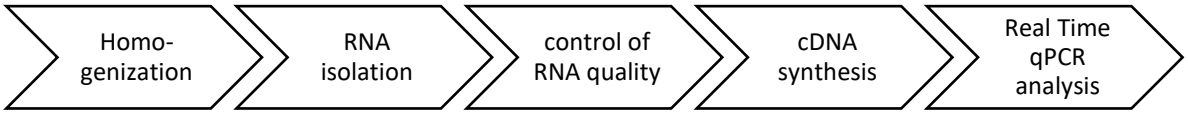

Figure 6. Schematic view on homogenization, RNA isolation, control of DNA quality, cDNA synthesis and qPCR analyses.

2.5.1 Homogenization

Gill samples on RNAlater were thawed on ice and 3-4 lamellae dissected and added to 1 ml RNAse free tubes containing 2 x 3 mm metal beads (Lysing Matrix S, 3.175 mm stainless steel beads, MP Biomedicals, USA). 400 µl of lysis buffer from the Agencourt® RNAdvance™ Tissue Kit (Beckman Coulter, USA) and 20 µl Proteinase K (Beckman Coulter) were added to each sample.

The tissue samples were homogenized using the FastPrep96 (MP Biomedicals) for 3 x (1800 rpm x 60 seconds) and spun down using a centrifuge (Avanti J-30-I, Beckman Coulter). 360 µl from homogenized samples were transferred to a new plate, and put in the heat incubator (TS 9000, Termaks, Germany) for 25 min at 37 °C. The tissue samples were stored at -80°C before RNA isolation.

2.5.2 Total RNA isolation

The total RNA was isolated using the Biomek 4000 robot (Beckman Coulter, USA). The samples were thawed in heat cupboard at 37 °C (Termaks), and then transferred to deep well plate. The method was conducted according to the manufacturer's instructions (RNAdvance Tissue Kit- Total RNA Isolation from Tissue, Beckman Coulter, US). In brief, five containers were filled with 1) 177.2 ml 70% EtOH (VWR, France) and 65.8 ml dH_2O, 2) 8.08 ml binding solution (Beckman Coulter) and 32.32 ml of isopropanol prima (Antibac AS, Norway), 3) 8.8 ml Nuclease-Free water (Nuc-H_2O) (HyClone, Utah, US), 1.1 ml DNAse I (Thermo Fisher Scientific) and 1.14 ml DNAse buffer (Thermo Fisher Scientific), 4) 160 ml wash buffer (Beckman Coulter) and 5) container with 5.2 ml with Nuc-H_2O (HyClone). The program took approximately 3 hours and included steps shown in figure 7:

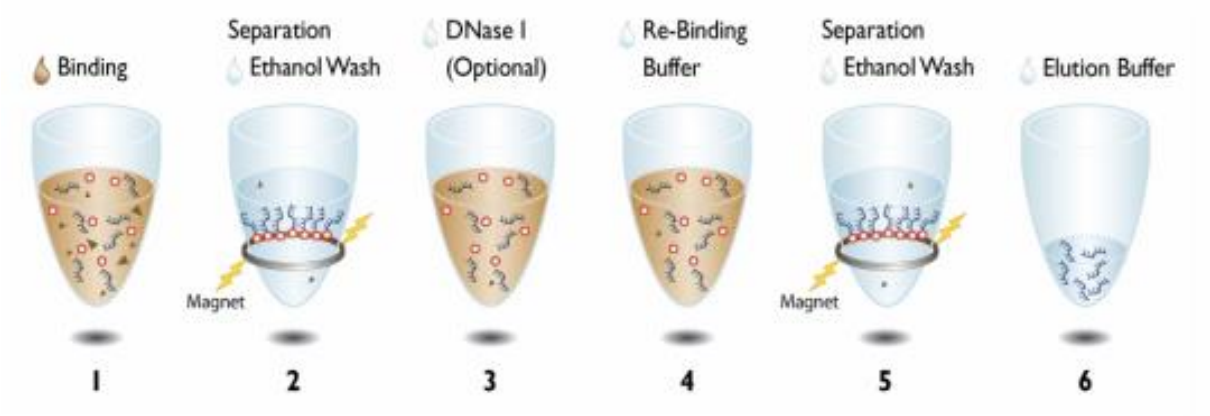

Figure 7. Schematic overview over the RNA isolation procedure using the Biomek 4000 robot. Binding solution (1) DNase I (3), wash buffer (4) and elution buffer (6) were added to the tissue samples, and between the procedure the samples were washed with washing buffer and ethanol (2), and then just ethanol (5). Figure from (Beckman Coulter, 2019).

Binding solution (Beckman Coulter) was added to the tissue samples which causes the magnetic beads to separate from the supernatant. The samples were washed with washing buffer (Beckman Coulter) and ethanol (VWR). DNase I was added to the samples (3), and then wash buffer (4). The magnet beads were again separated from the supernatant, and the samples were washed with ethanol (5). Elution buffer (Nuc-H_2O) (HyClone) was then added to the samples (6). This procedure`s product was extracted RNA, and the procedure was done twice in order to extract RNA from all the samples for this study (Beckman Coulter, 2019).

2.5.3 RNA-quality control

All RNA concentrations were checked using the NanoDrop8000 (Thermo Fisher Scientific). The pedestals on the NanoDrop were washed with dH_2O and lens paper. 1.6 µl RNA sample was placed on each pedestal. Values and graphs were checked before the results were transferred to an Excel document.

The NanoDrop instrument (Thermo Fisher Scientific) measures the absorption of the solution at a wavelength of 260 nm to determine the RNA concentration (ng/µl) in the solution. The instrument also measures the absorbance at 280 nm, where the ratio between the absorbance at 260 nm and 280 nm is used to determine the purity of the RNA. When the 260/280 ration has a ratio of ~2.0 it is considered an acceptable result. If the ratio is lower, it will indicate that the sample is contaminated with other substances that also absorb light at 280 nm.

After getting the RNA quality, a dilution plate of 22.2 ng/µl RNA was made in order to extract an equal amount of RNA for cDNA synthesis. The water:RNA ratio was calculated in excel to obtain an RNA concentration of 22.2 ng/µl. The samples were then diluted to a concentration of 22.2 ng/µl.

A selection of the samples was also analyzed using Bioanalyzer (Agilent 2100 Bioanalyzer, US) according to the manufacturer`s protocol to ensure good quality of the extracted RNA. RNase degradation of RNA samples is a common cause for failed experiments. The bioanalyzer system results in both gel-like images and electrophoretic data, which make it easy to detect small degrading effects. The system provides a RIN value (RNA Integrity Number), which provides a calculation of the total RNA quality in the samples. This scale ranges from 10 to 1, where RIN of 10 indicates highly intact RNA, while 1 indicates completely degraded RNA (Agilent, 2020).

Bioanalyzers were run on 6 of the gill samples to check whether the samples were degraded or not. The RIN had values between 9.7 – 10 indicating that the samples had highly intact RNA.

2.5.4 cDNA synthesis

In qPCR, cDNA is used as the template for the reaction since RNA is not a target molecule for DNA polymerase I. RNA must therefore be transformed into complementary DNA (cDNA) through reverse transcription. Isolated RNA is added to a master mix solution with the enzyme reverse transcriptase, as well as nucleotides and short primary sequences. Oligo dT-primer binds to the Poly A-tail of the mRNA, and the reverse transcriptase enzyme (MuLV) attaches to this region, and synthesizes a complementary DNA strand, resulting in a ss cDNA. The mRNA is degraded, DNA polymerase I binds to the ss cDNA, forming a complete ds cDNA which can then be used in the qPCR reaction (Saiki et al., 1988).

This entire process is divided into three active steps; primer activation, reverse transcription and mRNA degradation, with steps controlled by changes in temperature. In this study, "the High-Capacity RNA-to-cDNA™ kit" (Thermo Fisher Scientific) was used. The kit contains 1 x 500 µl of 2X RT buffer mix (dNTPs, random octamers and oligio dT-16), and 1 x 50 µl of 20X enzyme mix (MuLV and RNase inhibitor enzyme). To have enough master mix for the number of samples, 700 µl buffer mix and 70 µl enzyme mix were thawed and mixed to form a master mix. 11 µl of the master mix was transferred to wells on a 96-well plate. 9 µl of diluted RNA (22.2 ng/µl) was then transferred to the plate with master mix. This process yields 200 ng of RNA in the cDNA reaction. The process is done on ice during the entire time to prevent evaporation and degradation of the RNA. The plates were sealed and centrifuged to remove any air bubbles.

The reaction was done using a Thermocycler (Applied Biosystems 2700 Thermal Cycler, USA) with the following cycle parameters; 37 °C for 60 min, 95 °C for 5 min and then cDNA was stored at -20 °C.

cDNA was diluted 1:10 with nuclease free water; 20 µl cDNA + 180 µl H_2O. Furthermore, the cDNA (1:10), was again diluted 1:40 on a new plate; 50 µl cDNA (1:10) + 150 µl dH_2O (nuclease free water) to qPCR analyses.

2.5.5 qPCR analyses

qPCR is used to quantify the expression of specific genes from the gills. The method uses specific primers and DNA polymerase to amplify specific DNA sequences to a target gene. SYBR Green was used as a detection agent which non-specifically binds to double-stranded cDNA (ds cDNA) and emits strong fluorescence. The amount of ds cDNA doubles with each PCR cycle, and the fluorescence signal from the dye increases proportionally (Pfaffl, 2004).

When the fluorescence signal increases above the background, it will exclusively be the cDNA template for the specific primers that is amplified and doubled in an exponential phase. The cycle achieved is then called the cycle threshold (Ct) and is a relative expression of the number of cycles completed after the fluorescence signal hits the threshold value for amplification. The Ct value is inversely related to the amount of cDNA in the analyzed sample (Pfaffl, 2004).

In this study, qPCR was carried out using the Quant Studio 5 instrument (Applied Biosystems, Thermo Fisher Scientific, Singapore). Program for qPCR using SYBR Green is illustrated in table 1. Data was collected in QuantStudio™ Design & Analysis Software.

Table 1. Program for qPCR using SYBR Green.

Step	Temperature (°C)	Time (seconds)	Cycles
Hold (denaturation)	95	20	Hold-fase
PCR (denaturation)	95	1	40
Hybridization and polymerization	60	20	40

7 µl of cDNA (1:40) was distributed on 384 well plates (total of 7). The plates were then sealed, spun down and frozen at -20 °C. 7 µl Nuc-H_2O was added to the NTC (no template control) wells, 7 µl NRT (no reverse transcriptase control) to the NRT wells, and 7 µl positive control to two wells per primer/gene. A positive control was created by mixing 6 µl of sample from a total of 24 different wells (rows 1, 3, 6) from the plate with cDNA (1:40). A mix of many cDNA samples from the analyzed tissue should lead to a positive Ct value on the primer pair. The NTC control is a sample without a DNA/RNA template, which gives evidence of contamination in the sample set and a primer-dimer control using SYBR green. The NRT control is a control

without added enzyme in the cDNA reaction. If the NRT control is expressed, this will give an indication of contamination of genomic DNA in the samples.

qPCR master mix for the samples was made from PowerUp™ SYBR™ Green Master Mix (Thermofisher, Lithuania). 13 µl master mix was distributed to the relevant well with cDNA, in duplicates, but also to negative control and positive control. The amount of master mix for each primer pair is shown in table 2. The plate was then sealed and spun down before analysis.

Table 2. Overview of reagents used for master mix (per reaction and for full plate). Primers were diluted in dH_2O to a concentration of 5µM before used in the master mix.

1 x	**1 reaction**		**150 reactions for full plate**		
			160 reactions		
10	µl	SYBR green	1,6	ml	SYBR green
1,2	µl	Primer FW (5 µM)	192	µl	Primer FW (µM)
1,2	µl	Primer RW (5 µM)	192	µl	Primer RW (µM)
0,6	µl	Nuc-H_2O	96	µl	Nuc-H_2O
= 13 µl reaction mix + 7 µl sample					

Forward and reverse primers were prepared according to primers protocol (Invitrogen, Scotland, UK) to make a concentration of 100 µM, and further diluted to a working solution of 5 µM. The genes in this thesis have previously been published and used for Atlantic salmon and can be found in table 3 below.

Table 3. Sequences of oligonucleotide primer genes used in qPCR.

Function	Gene	Sequence (5'- 3')	GeneBank acc. no.
House-keeping gene	Ef1α	Fw: CGCCAACATGGGCTGG Rev: TCACACCATTGGCGTTACCA	DQ834870 (Olsvik et al., 2005)
House-keeping gene	β-actin	Fw: CAGCCCTCCTTCCTCGGTAT Rev: CGTCACACTTCATGATGGAGTTG	AF012125 (Olsvik et al., 2005)
House-keeping gene	18S	Fw: TGTGCCGCTAGAGGTGAAATT Rev: CGAACCTCCGACTTTCGTTCT	AJ427629 (Olsvik et al., 2005)
Stress	Hsp70	Fw: CCCCTGTCCCTGGGTATTG Rev: CACCAGGCTGGTTGTCTGAGT	BG933934 (Solberg et al., 2012)
Stress	Hsp90	Fw: CCACCATGGGCTACATGATG Rev: CCTTCACCGCCTTGTCATTC	>contig03769 length = 1183 numreads = 111 (Olsvik et al., 2013)
Stress (oxidative)	Cu/zn sod	Fw: CCACGTCCATGCCTTTGG Rev: TCAGCTGCTGCAGTCACGTT	BG936553 (Solberg et al., 2012)
Stress (oxidative)	Mn SOD	Fw: GTTTCTCTCCAGCCTGCTCTAAG Rev: CCGCTCTCCTTGTCGAAGC	DY718412 (Solberg et al., 2012)
Stress (oxidative)	GPx	Fw: GATTCGTTCCAAACTTCCTGCTA Rev: GCTCCCAGAACAGCCTGTTG	BG934453 (Solberg et al., 2012)
Immune response	il1β	Fw: CATCATCGCCATGGAGAGGTTA Rev: CAACTCCAACACTATATGTTCTTC	NM001123582 (Bakke et al., 2021)
Pain	trpv1	Fw: CGTCCTGCTGAAGGCTCTA Rev: TGTCTGTGTATGCAGCATTTACAA	NM001140498 (Nisembaum et al., 2022)
Apoptosis	casp3a	Fw:ACAGCAAAGAGCTAGAGGTCCAACAC Rev: AAAGCCAGGAGAGTTTGACGCAG	DQ008070 (Østbye et al., 2011)
Structurally gene	Claudin 10e	Fw: ATCAAGGTGGCCTGGTACTG Rev: GACCAGAGCACAGGGAAGTC	BK006391 (Tipsmark et al., 2008)
Structurally gene	Claudin 28a	Fw: TGACTGCTCAGGTCATCTGG Rev: GGTAAGGCCAGAAGGGAGTC	BK006401 (Sveen et al., 2018)
Structurally gene	Collagen 1a	Fw: CTCAGGGCTGCGGATGTT Rev: ACATGATGAACGACCGTGACA	XM014209886.2 (Ytteborg et al., 2010)

2.6 Histology

Histology was used to study morphological changes in the gills.

Gill samples were taken out of the formalin pots in a flume hood, cut into equal-sized pieces, and placed in an embedding cassette (Jiangsu Huida Medical, China) (figure 8A). The cassettes were marked with the project number, tissue, sample day and fish number using a printer (Signature cassette printer, Primera, USA). The cassettes with the gill samples were placed in a tissue processor (Logos Microwave Hybrid Tissue Processor, Milestone Medical, Italy) for dehydration and paraffin infiltration (figure 8B). The following program is shown in table 4.

Table 4. Program for the Logos Microwave Hybrid Tissue Processor for dehydration and infiltration of tissue.

Step	**Reagents**	**Time**
1	10 % formalin	Adapted machine
2	70 % ethanol	3 – 5 min
3	Absolute alcohol	55 – 60 min
4	99 % isopropanol	1.55 – 2.25 h
5	Paraffin wax	2.30 – 4 h

After dehydration, the cassettes with the gill samples were embedded with paraffin wax (Histowax, Histolab products AB, Sweden) using a heated paraffin embedding module (Leica EG1160, Leica biosystems, Nussloch, Germany). Embedding trays in metal were filled with warm paraffin wax on a hot plate (60°C) (figure 8C), and the gill samples were carefully placed in the middle part of the tray. The metal tray was then transferred to the cooling plate (Leica biosystems) (figure 8D), to make the wax harden and prevent the tissue sample from changing position. The cassette was then placed on top of the samples, pressed down, and a little extra paraffin was added (figure 8E). When all the wax had hardened, the metal tray and excess wax could be removed from the cassettes and the biopsies were ready for sectioning (figure 8F).

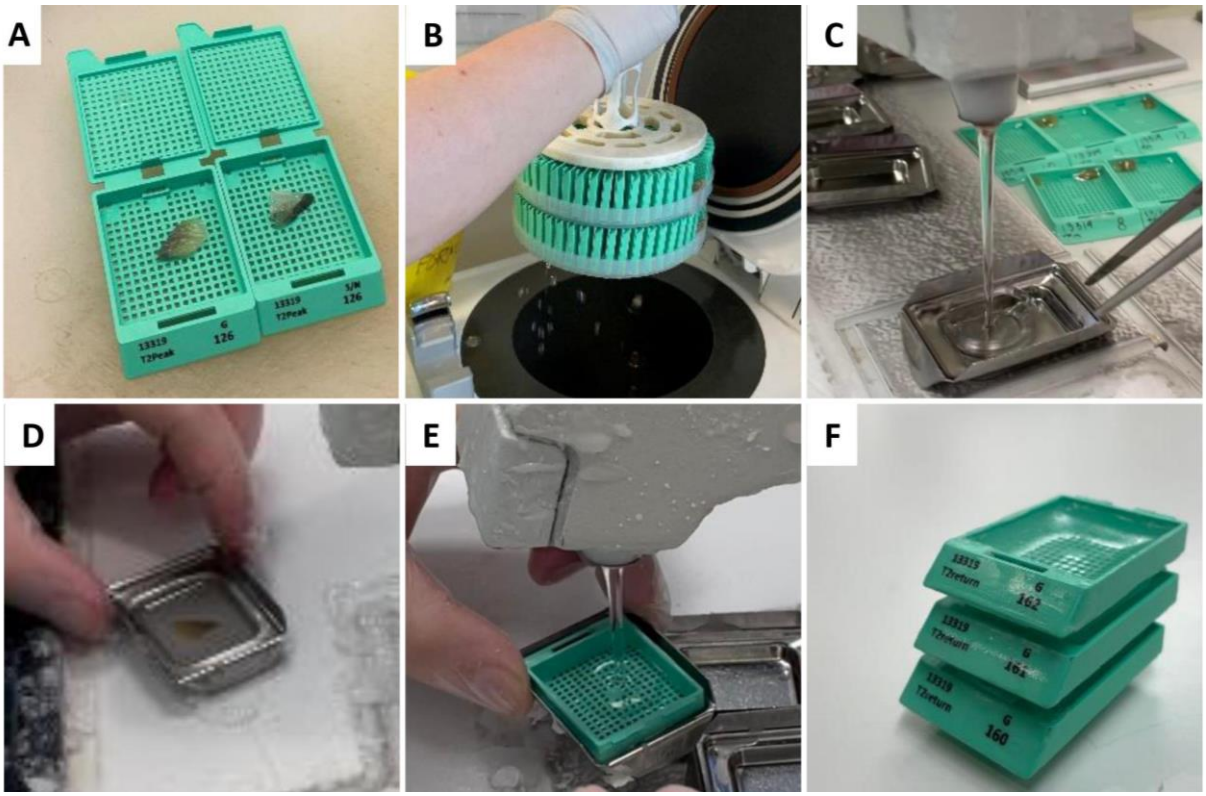

Figure 8. Paraffin infiltration and embedding of gill samples using tissue processor (Logos Microwave Hybrid Tissue Processor), heated paraffin embedding module and cooling plate (Leica EG1160). (A) Placing gill sample in embedding cassettes (Jiangsu Huida Medical). (B) Placing the samples in the tissue processor and (C) fill the embedding trays with paraffin wax (Histowax). (D) Transferring the tray to the cold plate and let the wax harden a bit before (E) fill up the tray with more wax. (F) Let the paraffin wax solidify and remove the embedding tray from the embedding cassettes so the samples become ready for sectioning.

The cassettes were put on ice, to harden the paraffin wax and make it easier to cut (figure 9A). Once cool, the biopsies were trimmed (18 µm) using a microtome (Leica RM2255, Leica Biosystems, China) to reach the tissue inside the paraffin wax. The biopsies were then cut into sections (2 µm thick) (figure 9B). Sections were placed in a heating bath at 43 °C (TFB 35 Tissue Flotation Baths, Medite, Burgdorf, Germany) (figure 9C). Sections with the tissue of interest picked up from the heating bath with labeled microslides (KP Silan Printer Slides, Klinipath, Netherlands). The slide with the tissue sample was air-dried at room temperature (23 °C) until the slide was dry. Throughout this process, it was important to keep it clean around the microtome to avoid large variables between sections.

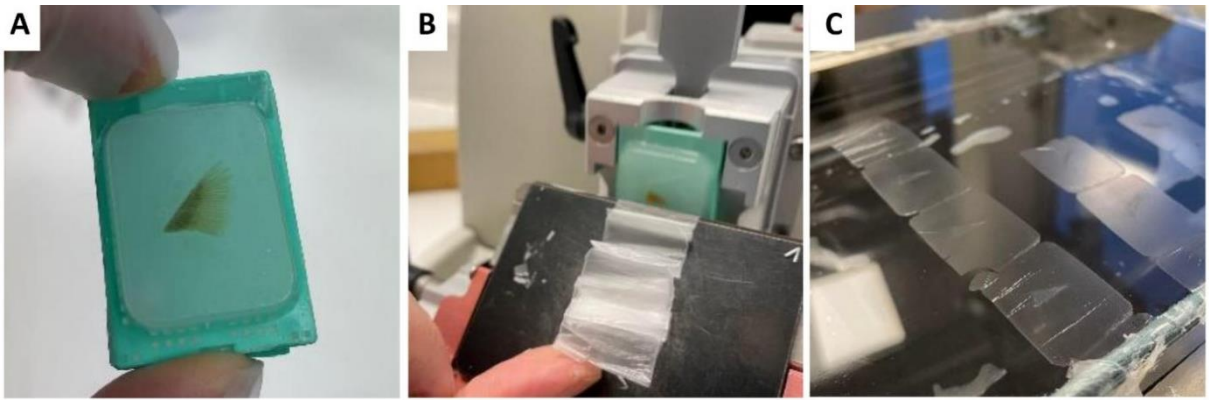

Figure 9. Sectioning of paraffin wax embedded tissue samples using microtome (Leica RM2255). (A) Hardened cassettes ready for sectioning. (B) Biopsies were first trimmed before cut into sections on 2 µm thick. (C) Sections were placed in heating bath before picked up with labeled microslides.

Alcian blue-Periodic acid-Schiff (AB-PAS) staining of the tissue samples was carried out using an automated staining robot (Tissue-Tek Prisma Plus, Sakura, Netherlands). The process contained several steps according to the protocol below (table 5). The protocol includes deparaffinization; where the slides are heated to 60°C to remove paraffin, rehydration; which is re-introduction of water to the tissue, and AB-PAS staining; which is the colour method itself.

Table 5. Protocol for Alcian blue-Periodic acid-Schiff (AB-PAS) staining. The process includes different steps (Layton & Bancroft, 2019) shown with colour coding: drying station (gray), deparaffinization (orange), rehydration (blue), wash with distilled water (white), staining (green), oxidation step (pink), dehydration (yellow) and clear.

Step	Solution	Time
1	Drying station (60 °C)	15 min
2	Xylene	30 sec
3	Xylene	4 min, 30 sec
4	Xylene	5 min
5	Absolute alcohol	2 min, 30 sec
6	Absolute alcohol	2 min, 30 sec
7	Alcohol 95%	45 sec
8	Alcohol 70%	45 sec
9	Wash	2 min
10	Alcian Blue 1% (Alcian blue in 3% aq. Acetic acid)	15 min
11	Wash station	2min
12	Periodic Acid (1% aq periodic acid)	10 min
13	Wash	10 sec
14	Schiff`s reagent	15 min
15	Wash	5 min
16	Hematoxylin	30 sec
17	Wash	1 min, 30 sec
18	Blaaning (Browsing)	1 min
19	Wash	1 min, 30 sec
20	Hydrochloric acid	2 sec
21	Wash	35 sec
22	Alcohol 70%	10 sec
23	Alcohol 95%	10 sec
24	Absolute alcohol	10 sec
25	Absolute alcohol	15 sec
26	Absolute alcohol	15 sec
27	Xylene	10 sec

After AB-PAS staining the slides were then covered with an automated cover slipper (Tissue-Tek Film, Sakura, USA) (figure 10A), and then scanned into a PC (figure 10C) using a slide scanner (Nano Zoomer S360, Hamamatsu, Japan) (figure 10B).

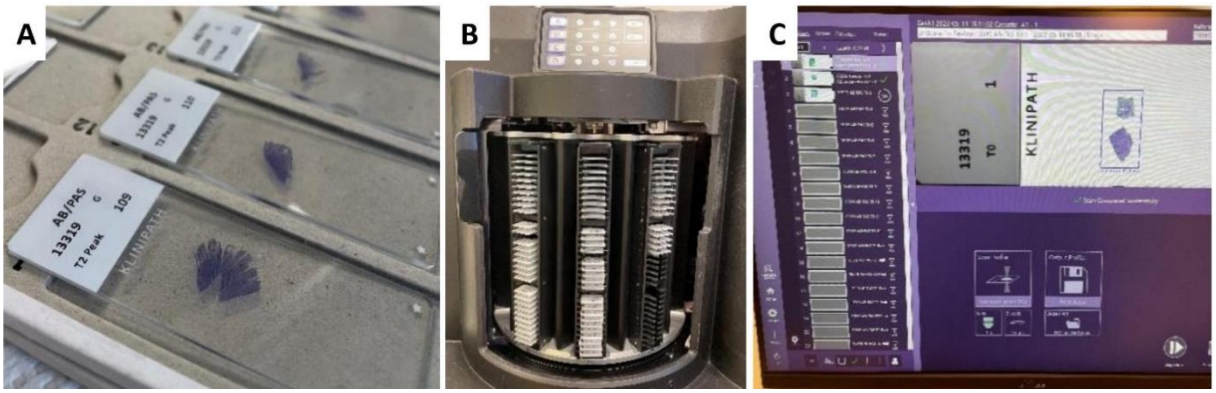

Figure 10. (A) Slides covered with cover slipper Tissue-Tek Film before (B) scanned with Nano Zoomer (C) into a PC program.

2.7 Gill scoring

Histological analyses were performed on gills (from AB-PAS staining) to evaluate structural changes between the different temperature regimes. Both manual observations and digital measurements of morphological and histopathological changes were included, such as clubbing, mucous cell presence and aneurism occurrence. Previous studies have shown that a combination of manual measurements with the digital scoring platform Aiforia® has highly correlated and provided good results (Sveen et al., 2021).

2.7.1 Manual scoring system

A gill scoring system modified from Mitchell et al., (2012) was used for histological evaluation, to score inflammatory reactions, damages and morphological deviation. All samples were analyzed in QuPath (v.0.3.2), and two rounds of scoring were carried out by two individuals before the scores were compared to each other. Histological evaluation of gill sections is subjective, and the credibility of the analysis depends on, among other factors, the experience of the histologist, the type of scoring system used and the quality of the samples (Wolf et al., 2015). The scoring system was therefore prepared together with experienced researchers, based

on several already published gill scoring systems and modified from established systems (Lazado et al., 2022; Mitchell et al., 2012).

Using the scoring system, the quality and health status of gills are assessed based on various criteria. A brief description of the criteria: criteria 1 is based on the severity and presence of fusion in the secondary lamellae, criteria 2 presence and size of clubbing, criteria 3 the presence, position and colour of mucous cells, criteria 4 presence of aneurism and criteria 5 lifting presence.

For each criteria, a score from 0-4 is given, where 0 is no change, while 4 is severe change. In order to set a score, a description is given for each morphological change for each score. A score of 0-1 indicates no or mild change and can be considered normal morphology. Score 2 indicates moderate changes, score 3 distinct changes and a score of 4 will indicate severe morphological changes. The samples were analyzed blind, which means that information about the fish and the treatment it was subjected to was not known while the scoring was carried out. This may help to ensure that there is no preconceived notion of what the result should be in terms of hypotheses and expected results.

In the upcoming tables, table 6 to 10 the various criteria are described. The scoring setup is inspired by a previous scoring description for the gastrointestinal tract (Johansson, 2014).

Fusions were divided into two subdivisions, one for the secondary lamella fusion severity and one for fusion presence (table 6). In the first subdivision, severity, the gills are scored from the number of secondary lamellae that has fused together. The lowest score indicates no cases of fusion between the secondary lamellae, and increased number of secondary lamellae that has fused together is indicated by higher scores. In the other subdivision, presence, the number of cases of fusion is scored. Score 0 indicates no cases of fusion between secondary lamellae, and higher scores indicate increased presence of fusions. Examples of fusion severity in secondary lamellae are presented in figure 11.

Table 6. Scoring criteria for fusion in secondary lamellae.

Criteria 1 - Fusion	Score	Description	Appearance
Severity	0	None	No fusions of secondary lamellae
	1	2 fused	Two secondary lamellae have fused
	2	3-4 fused	Three or four secondary lamellae have fused
	3	Several	Several secondary lamellae have fused
	4	Massive	Nearly all secondary lamellae have fused
Presence	0	None	No presence of fusions
	1	1-2 cases	One to two cases of fusions
	2	5-10 cases	Five to ten cases of fusions
	3	Several	Several cases of fusions
	4	All over	Nearly all secondary lamellae have fused

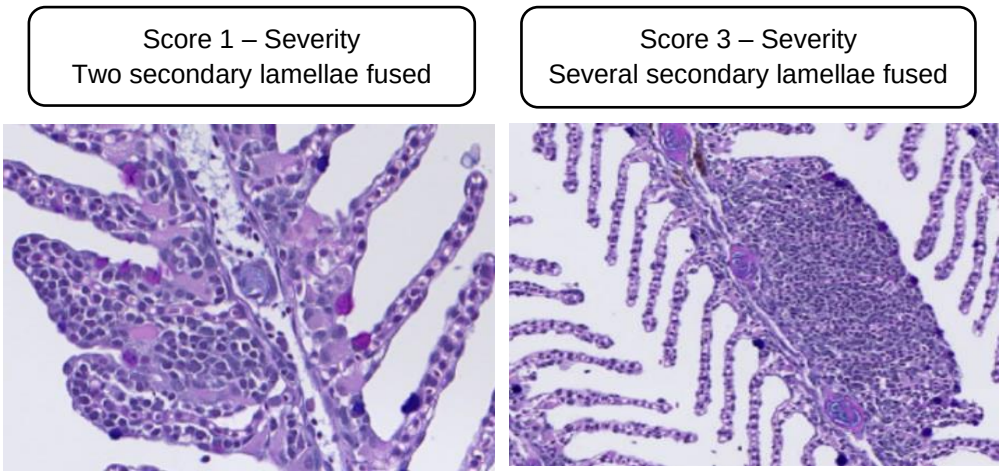

Figure 11. Examples of different scores of criteria 1 (subdivision severity) of Atlantic salmon gills. In normal gills the secondary lamellae are clearly separated from each other. Samples stained with Alcian blue-Periodic acid-Schiff (AB-PAS).

Clubbing was also divided into two subdivisions, one for clubbing presence and the other for clubbing size, table 7. Score 0 in clubbing presence indicates no presence of clubbing in secondary lamellae, and higher score indicate higher presence of clubbing. Examples of clubbing size in secondary lamellae are presented in figure 12.

Table 7. Scoring criteria for clubbing in secondary lamellae.

Criteria 2 - Clubbing	Score	Description	Appearance
Presence	0	None	No presence of clubbing on the secondary lamellae
	1	10%	Approximately 10% of secondary lamellae have clubbing
	2	50%	Approximately 50% of secondary lamellae have clubbing
	3	75%	Approximately 75% of secondary lamellae have clubbing
	4	100%	All secondary lamellae have clubbing
Size	0	None	No indication of clubbing
	1	Clear indications	A clear indication of clubbing
	2	Doubled	A doubling in cellular size of the secondary lamellae
	3	4 x enlarged	A quadruple in cellular size of the secondary lamellae
	4	Massive	Massiv hyperplasia of secondary lamellae

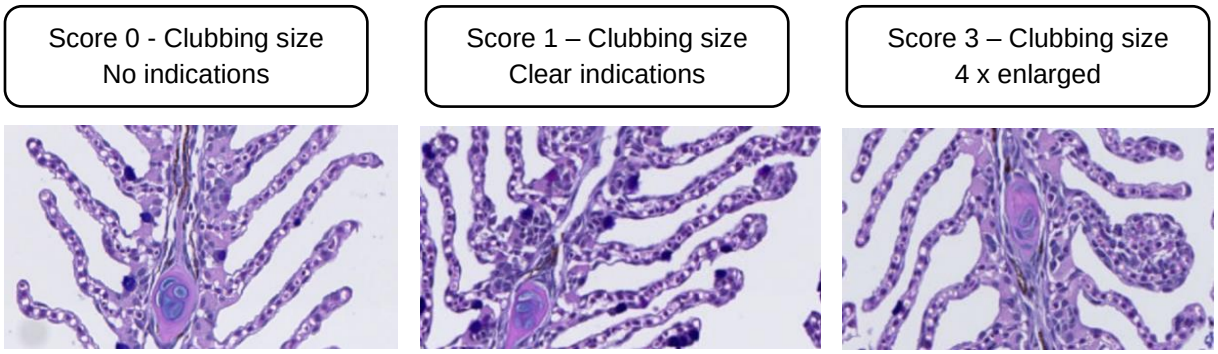

Figure 12. Examples of different scores of criteria 2 (subdivision clubbing size) of Atlantic salmon gills. In normal gills the secondary lamellae have no or minor indication of clubbing, hyperplasia. Samples stained with Alcian blue-Periodic acid-Schiff (AB-PAS).

A scoring system for different categorizations of mucous cells was included and divided into three subdivisions; one for mucous cells presence in the gills, one for their position and one for what kind of colour the mucous cells have (table 8). In AB-PAS staining mucous cells are often characterized with dark blue colour, depending on whether it is sour or neutral mucus (Smith et al., 2018). Examples of mucous cells presence and position are presented in figure 13.

Table 8. Scoring criteria for mucous cells in primary and secondary lamellae.

Criteria 3 – Mucous cells	Score	Description	Appearance
Presence	0	None	No mucous cells
	1	Sporadic	Sporadic mucous cells
	2	Evenly distributed, but few	Evenly distributed mucous cells, but they are few in numbers
	3	Evenly distributed, many	Evenly distributed mucous cells, many in numbers
	4	All over	Extensive amounts of mucous cells
Position	0	Primary lamella	Mucous cells are mainly in primary lamellae
	1	Secondary lamella	Mucous cells are mainly in secondary lamellae
	2	75% on the primary, 25% on secondary lamella	About 75% of all mucouse cells observed in primary lamellae
	3	50/50 on primary/ secondary lamellae	Equal amounts of mucous cells observed in the secondary lamellae as in the primary lamellae.
	4	None	No mucous cells in the gill
Colour	0	Dark blue only	All mucous cells are dark blue
	1	Pink only	All mucous cells are pink
	2	50/50 mix	50% dark blue/pink.
	3	75/25 dark blue	75% of mucous cells are dark blue, 25% pink
	4	90/10 dark blue	90% of the mucous cells are dark blue, 10% pink

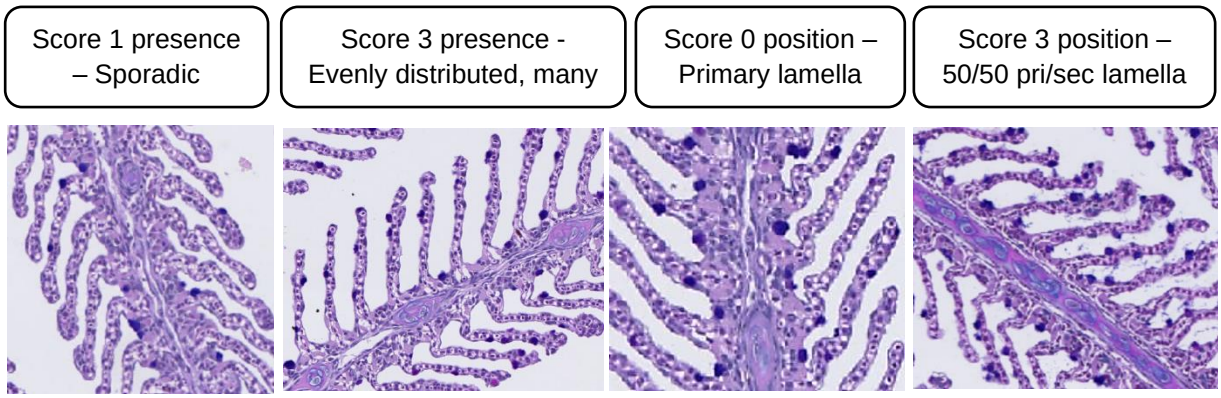

Figure 13. Examples of different scores of criteria 3 of Atlantic salmon gills. The two figures on the left show scores from mucous cell presence, while the two figures on the right show examples of the mucous cell's position in the gills. Samples stained with Alcian blue-Periodic acid-Schiff (AB-PAS).

Aneurisms (Criteria 4) characterize the presence of bleeding, aneurisms, in the gills, table 9. A gill with an increasing presence of aneurisms will get an increasing score. Example of aneurism presence in secondary lamellae is presented in figure 14.

Table 9. Scoring criteria for aneurisms in secondary lamellae.

Criteria 4 – Aneurism	Score	Description	Appearance
Presence	0	None	No aneurism in the gill
	1	Few, sporadic	Few, sporadic aneurisms detected
	2	Evenly distributed, but few	Aneurisms evenly distributed, but few in numbers
	3	Evenly distributed, many	Aneurisms evenly distributed, many in numbers
	4	All over	Extensive presence of aneurisms

Score 2 – evenly distributed, but few

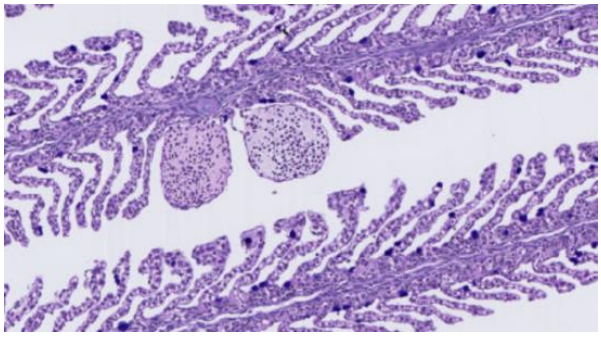

Figure 14. Example of criteria 4 in Atlantic salmon gills. The figure shows score 2 in aneurism presence in secondary lamella. Samples stained with Alcian blue-Periodic acid-Schiff (AB-PAS).

Lifting (Criteria 5) characterize the presence of lifting in the gills, table 10. Lifting is a term that refers to a condition in the gills where a clear gap has developed between the epithelial cells and beneath tissues, i.e., pillar cells and blood capillaries. Score 0 in presence indicates no presence of lifting in primary and secondary lamellae. A higher score indicates higher presence of lifting, where a score of 4 indicates that the whole gill has lifting present. Examples of lifting are presented in figure 15.

Table 10. Scoring criteria for lifting in primary and secondary lamellae.

Criteria 5 – Lifting	Score	Description	Appearance
Presence	0	None	No lifting is presented
	1	Less than 10%	Minor part of the gill has lifting
	2	Ca. 30%	There is a moderate presence of lifting
	3	More than 50%	More than half of the gill has lifting presence
	4	100%	The whole gill has lifting present

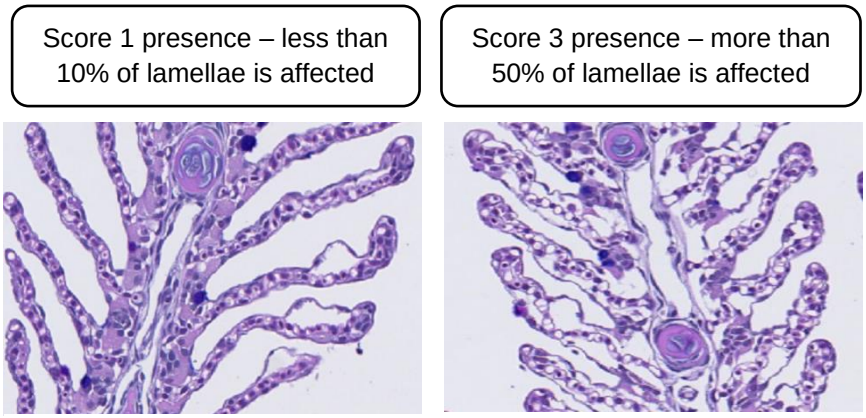

Figure 15. Example of different scores of criteria 5 of Atlantic salmon gills. In normal gills no lifting is observed. Samples stained with Alcian blue-Periodic acid-Schiff (AB-PAS).

2.7.2 Digital histopathology – Aiforia®

Digital analysis of AB/PAS-stained sections were done using a gill algorithm in the Aiforia® platform (Fimmic Oy, Helsinki Finland) developed by Nofima. The Aiforia platform is trained to identify and count different structures on tissue sections (figure 16), and the method has previously been used to analyze skin samples from Atlantic salmon (Sveen et al., 2021). Aiforia can show the development of clubbing, the percentage presence of mucous cells, as well as the number of blue mucous cells in the primary lamellae and secondary lamellae, in addition to the amount of chloride cells and aneurisms in the primary lamellae. The length of the secondary lamellae in the various temperature groups is also included in this scoring. A total of 111 out of 120 digitized gill sections were uploaded to the Aiforia®. Nine samples were excluded from the analysis due to artefacts. The digital analysis was intended as a support to the already presented manual scoring as well as to provide more data.

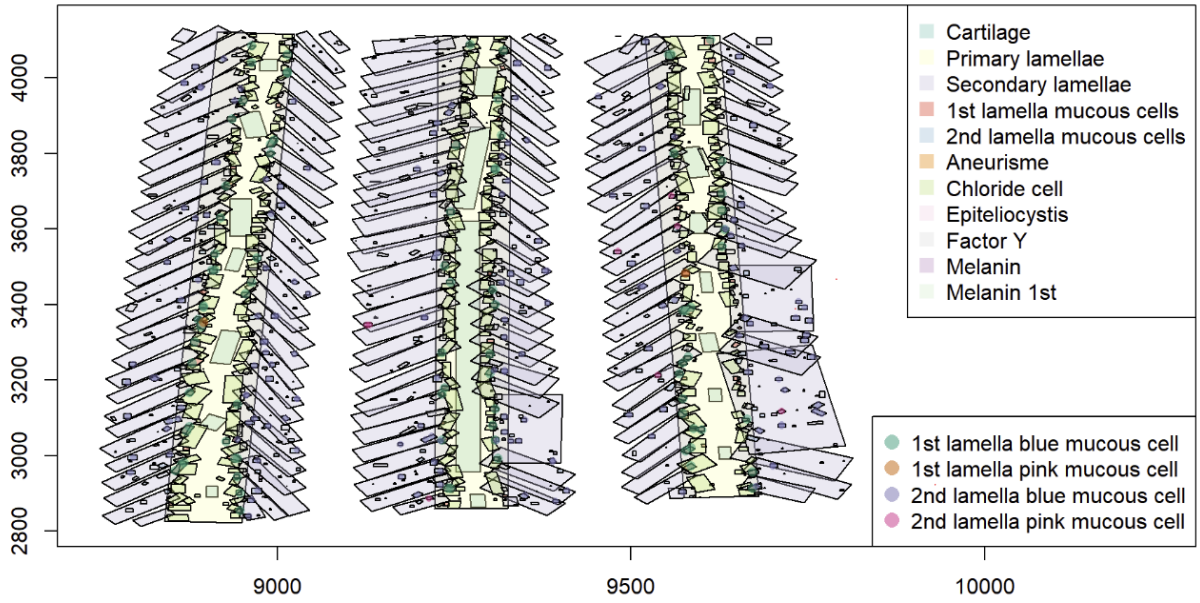

Figure 16. Overview plot of tissue sections and cell types in different colour codes the Aiforia platform® is trained to recognize.

The regions of interest (ROIs) were manually drawn on each tissue section (figure 17A). In this study, it was desirable to include regions with three primary lamellae together with secondary lamellae in the ROI, but on eleven sections only one primary lamella was included due to artefacts. Different colour overlayers represents tissue and cell detection. The AI-analysis detect the primary lamellae (pri, green), the secondary lamellae (sec, pink) and cartilage (car, purple) (figure 17B). Further detection of the cells in the gill includes mucous cells marked by light blue for mucous cells in the secondary lamellae and yellow for mucous cells in the primary lamellae (figure 17C). The AI-analysis was also used to separate between pink (figure 17D) and dark blue mucous cells (figure 17E). Chloride cells are marked by red circles (figure 17F).

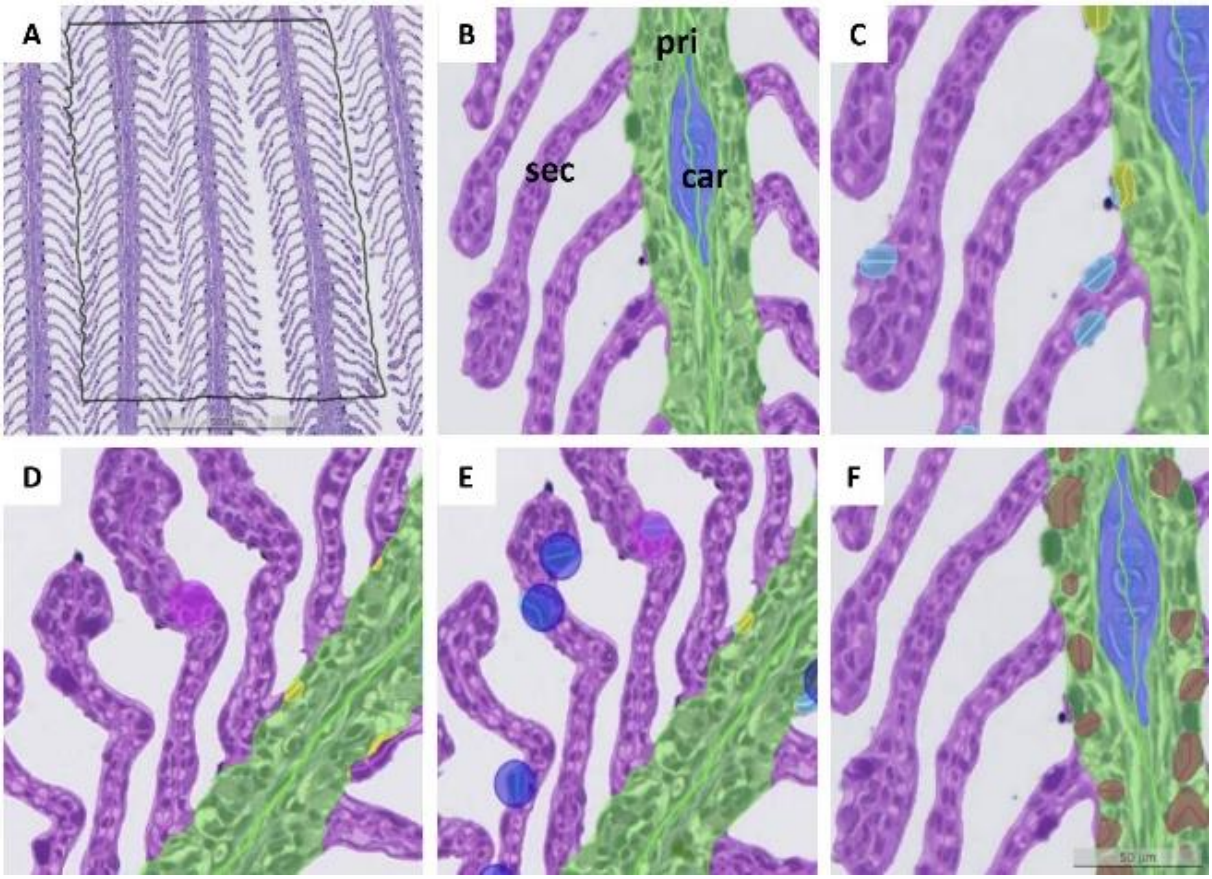

Figure 17. (A) The regions of interest (ROI) were manually drawn on each tissue section. (B) Details of tissue detection, primary lamellae (pri, green), secondary lamellae (sec, pink) and cartilage (car, purple). (C) Further detection of the different cells in the gill included mucous cells in the primary- and secondary lamellae, where (D) pink mucous cells and (E) blue mucous cells were identified. (F) Areas with chloride cells marked with red circles.

2.8 Data analyses and statistics

Statistics were performed in Microsoft Excel, unless stated otherwise. Results from the qPCR analyses were collected by the Quant Studio™ Design and Analysis software v.2.5.0 (DA2), where the threshold was adjusted manually to 0.1.

2.8.1 Aiforia® and the R-Studio software (R.4.0.2)

Results from Aiforia® were visualized inspected and statistically analyzed using the R-Studio software (R.4.0.2) by researchers at Nofima. For each region of interests (ROI) in Aiforia the platform produces a table, containing the predicted features based on the previously trained deep-learning model. An R-script was used to apply a quality filter for the different features and sort them into files for further analysis. These filtered data were then statistically analyzed for differences between groups. In the figures, the boxes illustrate 50% of the values that are closest to the median and the blue horizontal lines indicate the group means with their respective ± standard error of the mean (Nisembaum et al., 2022) as vertical lines. Three-way ANOVA was run in *R* to find significant differences ($p \leq 0.05$) in time point, group and temperature treatment. In case the ANOVA indicated significant differences, a Tukey's post-hoc test was run between the groups. Groups were marked with lower case letters (a, b, c ,d) and groups that did not share a letter were significantly different ($p \leq 0.05$).

Manual histology scores were treated as categorical data and were analyzed using the frequency analysis method Chi-Square (p-value set to ≤ 0.05). Statistics were run between each temperature group within the different time points.

2.8.2 Statistics of qPCR results

The melting curve signal was checked in the Quant Studio™, where melting peak with just one peak were accepted. Good signal on melting curve was around 80-85 °C. The ΔΔCt method was used to calculate the relative expression of genes.

Sigmaplot (v.14) was used to perform two-way ANOVA (analysis of variance) on the qPCR results, but also on weight, length, and condition factor. This statistical method was used to find out if there were significant differences between the sampling time point and/or between the different temperature groups. JMP® Statistical Software (v.16.2) was used to perform two-way ANOVA on TGC and SGR.

The two-way ANOVA is done after a normality test (Shapiro-Wilk) and equal variance test (Brown-Forsythe), using a pairwise multiple comparison procedure (Holm-Sidak method) which has a generally significant level of 0.05. In cases where the data material was not approved for the Holm-Sidak method, as for testing *il1β*, Duncan's method was used instead. Before two-way ANOVA, the data was checked for significant outliers, and these were removed from the rest of the samples using Grubb's test.

3. Results

3.1 Growth

Weight with ±SD of fish in different temperature groups are summarized in figure 18. The groups had a significant weight gain throughout the trial period. No significant difference between the temperature group's average weight was observed, except for Return 2 were there is a significant difference between the fish in control temperature (CT) and the fluctuating temperature (FT).

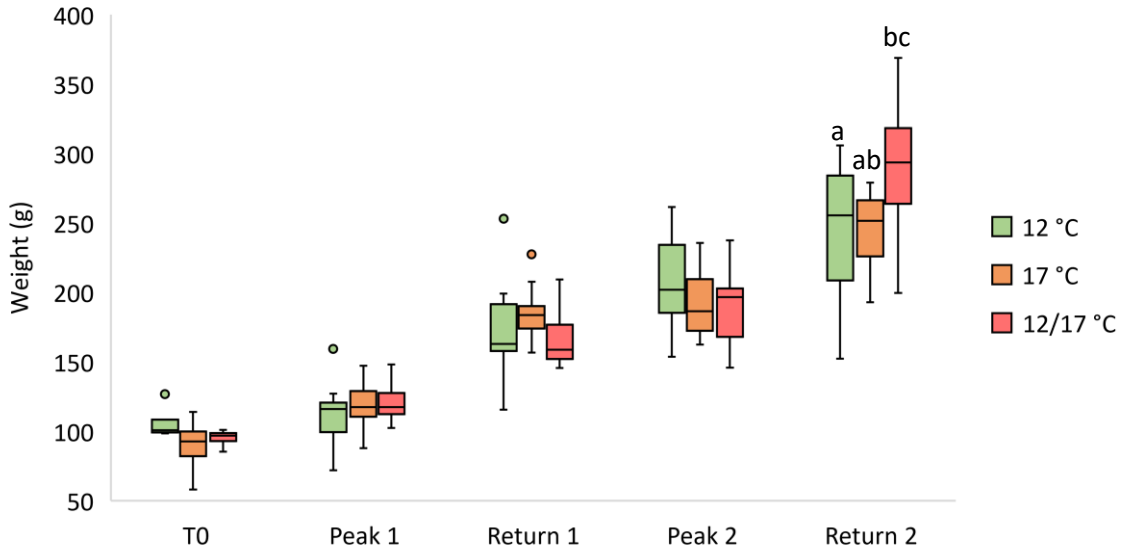

Figure 18. Weight (g) gain with ±SD for all the fish (n=10) in the different temperature groups on the different time points. Dots illustrate weight for fish that were outliers and different letters indicate significant differences among the groups at a particular sampling point ($p \leq 0.05$).

Length data with ±SD for each sampling is shown in figure 19. The different fish groups had a significant increase in length throughout the trial period, except for no significant length increase within CT between Peak 2 and Return 2, and in fish from high-temperature (HT) and FT between time point Return 1 and Peak 2. There was no significant difference observed between the temperature groups except for at Return 2, where the FT fish was significant longer than in CT and HT.

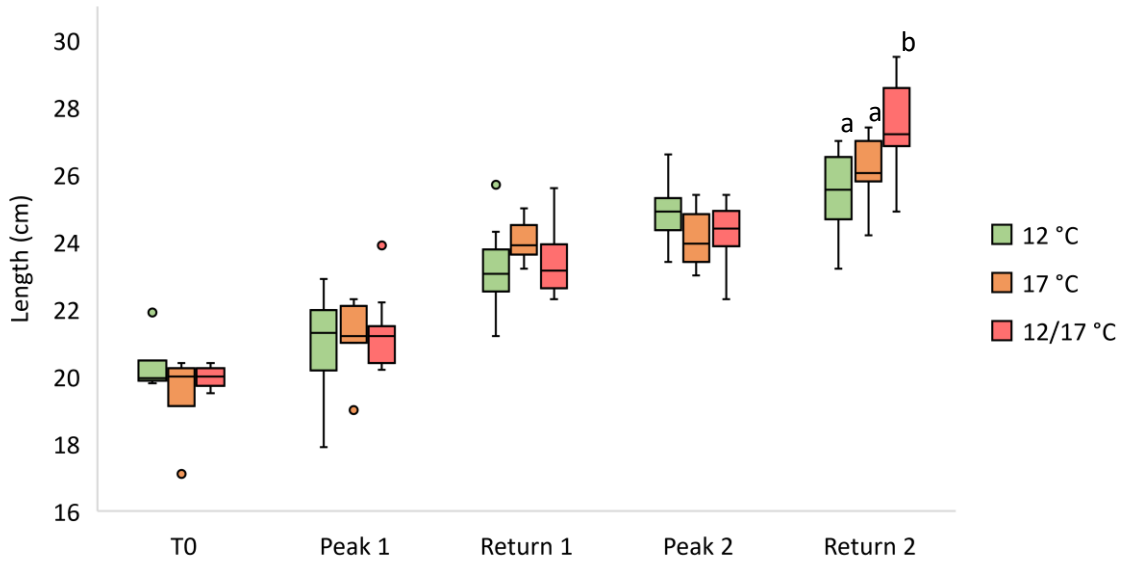

Figure 19. Length data (cm) with ±SD for all the fish (n=10) in the different temperature groups on the different time points. Dots illustrate length for fish that were outliers and different letters indicate significant differences among the groups at a particular sampling point ($p \leq 0.05$).

Development in condition factor (CF) is shown in figure 20. There were no significant differences in CF between the temperature groups.

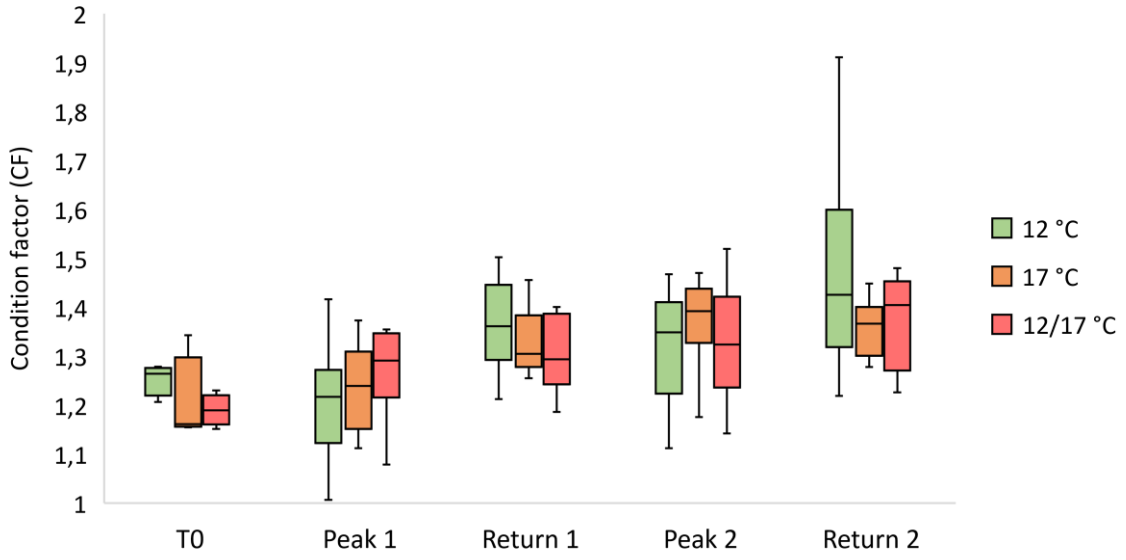

Figure 20. Condition factor (CF) with ±SD for all the fish (n=10) in the different temperature groups on the different time points. Dots illustrate condition factor for fish that were outliers and different letters indicate significant differences among the groups at a particular sampling point ($p \leq 0.05$).

TGC and SGR is shown in figure 21. There were no significant differences in TGC and SGR between the temperature groups.

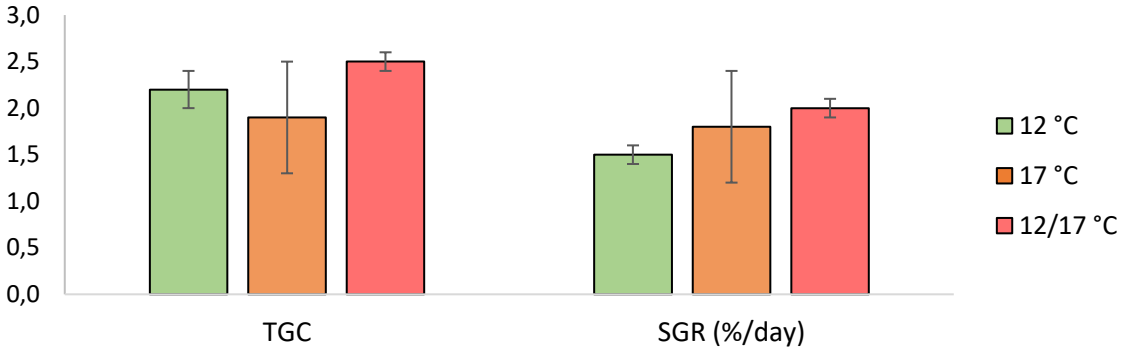

Figure 21. TGC and SGR (% / day) for the different temperature groups from T0 to Return 2. Different letters indicate significant differences ($p \leq 0.05$).

3.2 Histological analyses

3.2.1 Manual scoring of gill-samples

Gills were analyzed according to the scoring system presented in table 6 to 10. Gill score in Atlantic salmon post-smolt in the different temperatures, on different time points, during the trial period, is shown in figure 22. The gill scores are based on average damage score and showed a significant histological change in HT group and FT group, compared to the gills exposed for CT who displayed a general normal morphology.

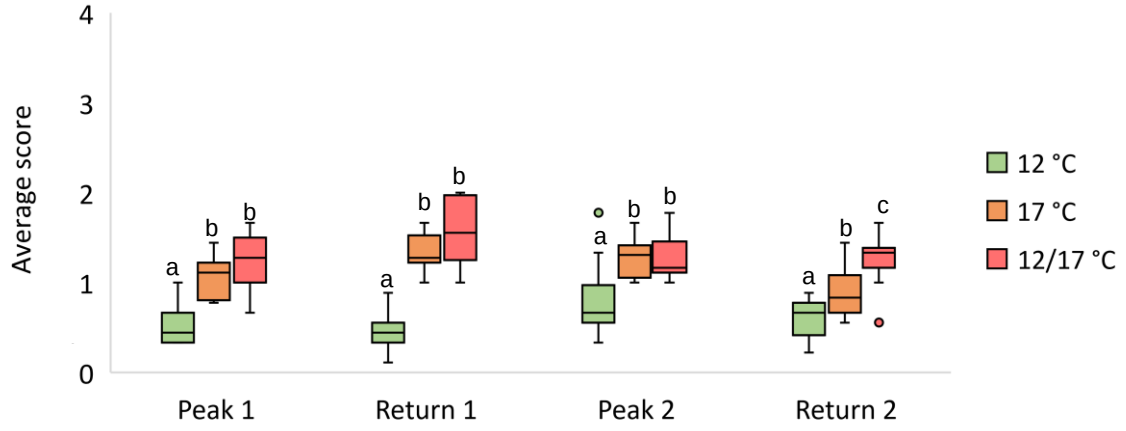

Figure 22. The distribution of the manual gill scores. Shows a clearly higher overall score for the FT group and the HT group compared to the CT. Different letters indicate significant differences among the groups at a particular sampling point ($p \leq 0.05$).

In figure 23, representative photographs are shown of A) healthy gills from CT (12 °C); clearly defined histological structures with normal gill morphology with clearly separated secondary lamellae, no fusion, hyperplasia or clubbing. B) gills from HT (17 °C) with the most common changes seen in this temperature group; clubbing, increased presence of mucous cells in secondary lamellae and started lamellar fusion. C) gills from FT (12/17 °C) with the most common changes found in these gills; clubbing, mucous cells in both primary and secondary lamellae and lifting presence. All three photos are collected from Return 1.

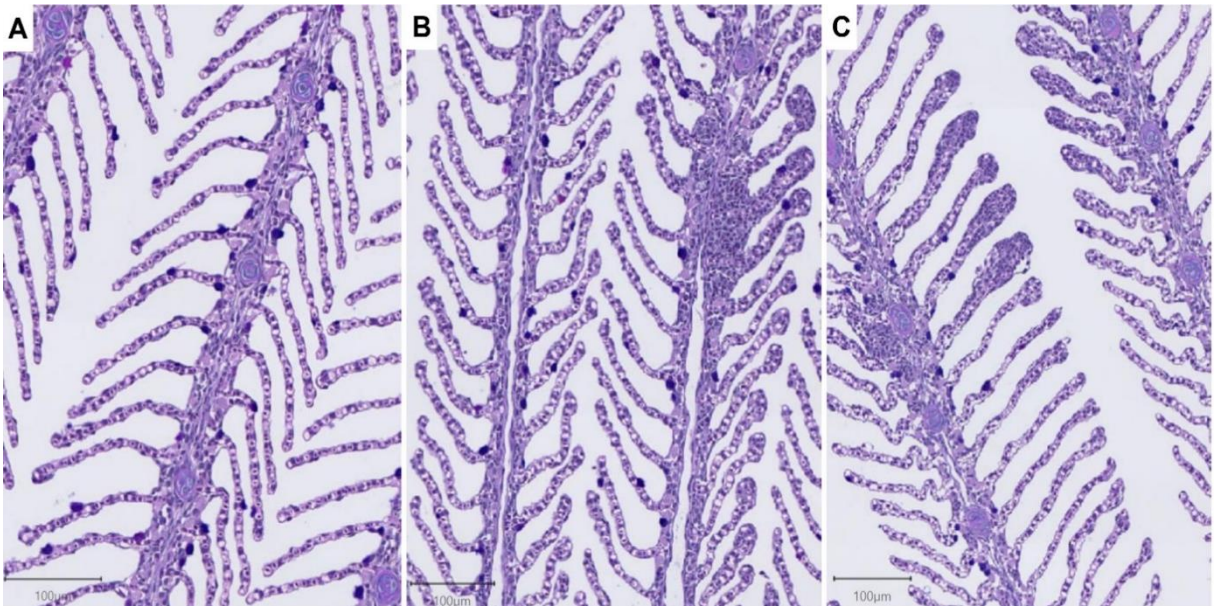

Figure 23. Representative photographs of (A) healthy gills in the control group, (B) the most common histological changes in the 17 °C group, and (C) in fluctuating group (12/17 °C), collected from Return 1. Samples stained with Alcian blue-Periodic acid-Schiff (AB-PAS).

Fusions

The score for criteria 1 (Fusions) with subdivision is showed in figure 24 and figure 25. Fusion severity (figure 24) shows there were significant differences between the temperature groups in Return 1 and Return 2. 80% of the gills from CT had no fusion and 20% with a score of 1, where two secondary lamellae were fused. Gills from HT had score 1 (15%), score 2 (65%) and score 3 (20%). Gills from FT were mainly given a score of 3 (80%). Return 2 shows that HT and FT group were significantly different from each other, where HT had 90% of score 0 and FT had 20% with score 0, 60% with score 1 and 20% of score 2.

The same development is seen for the presence of fusions (figure 25), where there were significant differences between gills from CT, HT and FT in Return 1 and Return 2. In Return

1, 80% of gills from CT had score 0 and 20% score 1. Gills from HT had 80% of score 1 and 20% of score 2. Gills from FT had 10% of score 0, 50% of score 1, 10% of score 2 and 20% of score 3. While in Return 2, only gills from HT and FT were significantly different from each other. Gills at HT had 90% of score 0 and 10% score 1, however gills from FT had 20% of score 0 and 80% with score of 1.

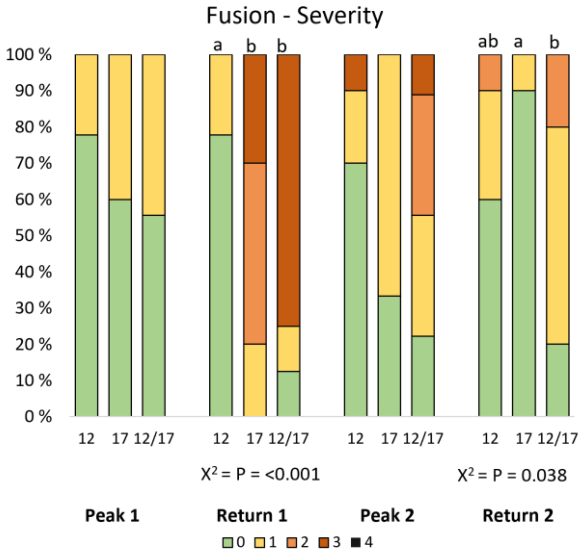

Figure 24. Histological analyses from criteria 1, fusion severity, of secondary lamellae from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates no fusion, score 1 shows that there were two secondary lamellae fused, score 2 was given samples with three or four secondary lamellae fused, score 3 when several secondary lamellae were fused, and score 4 was given when nearly all secondary lamellae were fused. Different letters indicate significance ($p \leq 0.05$).

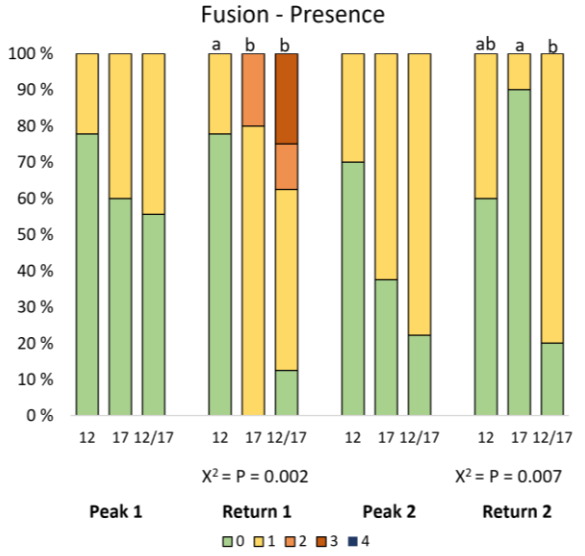

Figure 25. Histological analyses from criteria 1, fusion presence, of secondary lamellae from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates no fusion, score 1 given samples with one or two cases of fusion, score 2 when five to ten cases, score 3 when several cases were present, and score 4 was given when there was presence of fusion all over the gill. Different letters indicate significance ($p \leq 0.05$).

Clubbing

There was a generally greater presence of clubbing in gills from FT, compared to the gills from CT and HT (figure 26). In Peak 1 gills from CT had 100% of score 2, HT had 70% of score 2 and FT had a higher percentage of score 3 (70%). In Return 1 the same trend is shown, where gills from HT and FT had a high percentage of score 3 (70% and 60%), while the fish from CT doesn't have gills with score 3. In Return 2 gills from HT (10% of score 3) and FT (70% of score 3) were significant different from each other. The clubbed lamellae size was also significantly different between the temperature groups (figure 27), where the CT had a lower percentage of score 1, score 2 and score 3 compared to the gills exposed for HT and FT. As shown on the figure, gills from CT mostly had a score of 0 (figure 27).

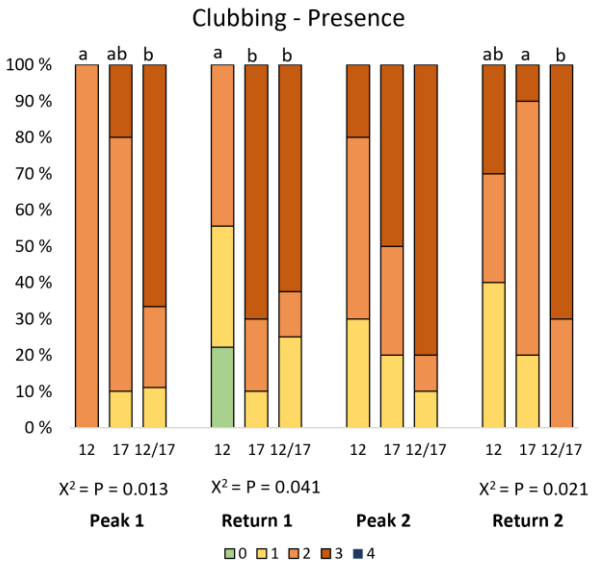

Figure 26. Histological analyses from criteria 2, clubbing presence, of secondary lamellae from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates no clubbing, score 1 given samples with 10% clubbing, score 2 when 50% clubbing present, score 3 when 75% clubbing present, and score 4 all secondary lamellae have clubbing. Different letters indicate significance ($p \leq 0.05$).

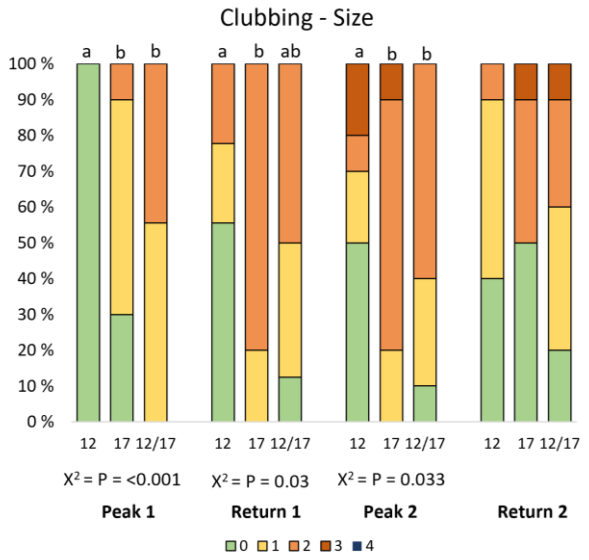

Figure 27. Histological analyses from criteria 2, clubbing size, of secondary lamellae from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates no clubbing, score 1 when clear indications, score 2 when doubled size, score 3 when the club has a quadruple in cellular size, and score 4 when massive hyperplasia. Different letters indicate significance ($p \leq 0.05$).

Mucous cell

The percentage of mucous cells presence was significantly different between the temperature groups in Return 2. Gills from the CT had the lowest presence of mucous cells, with a 100% of score 1, while HT and FT had 50% of score 1, and 50% of score 2 (figure 28). This was generally observed throughout the trial. Also, the mucous cells position showed significant differences between gills from CT, the HT and the FT in Peak 1 and Return 1 (figure 29). HT and FT had several gills with position score 2, i.e., mucous cells, not only in the primary lamellae, as for score 0, but also in the secondary lamellae.

For the mucous cells colour score there was a significant difference between gills from CT and HT in Peak 2 (figure 30). There were more pink mucous cells in the HT (40% of score 2) compared to CT, which mainly had dark blue mucous cells (90% of score 0) throughout the trial. In general, the gills from the CT had more of score 0 compared to HT and FT.

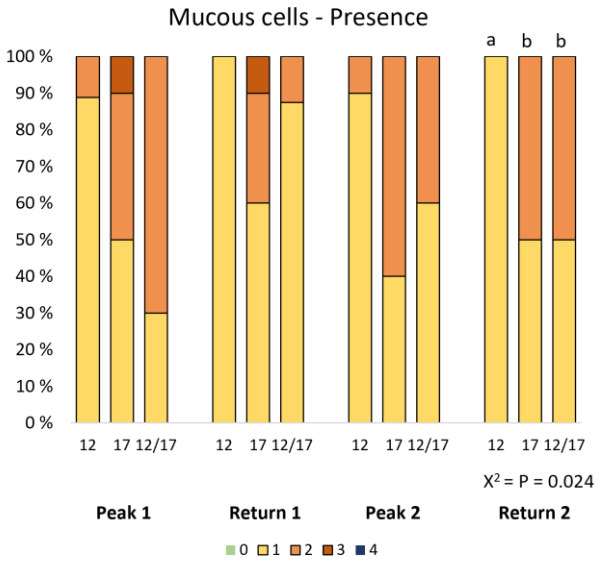

Figure 28. Histological analyses from criteria 3, mucous cells presence, in gill from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates no mucous cells, score 1 when sporadic present, score 2 when evenly distributed – few, score 3 when evenly distribute – many and score 4 when present all over the sample. Different letters indicate significance ($p \leq 0.05$).

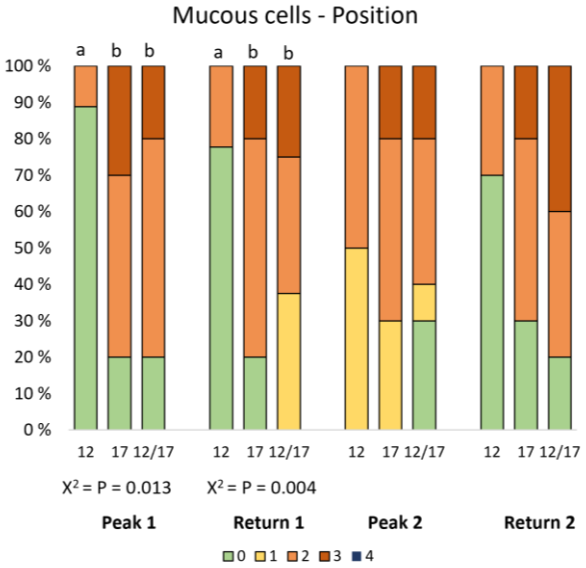

Figure 29. Histological analyses from criteria 3, mucous cells position, in gill from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates primary lamellae, score 1 secondary lamellae, score 2 when 75% are observed in primary lamellae, score 3 when 50/50 on primary/secondary lamellae and score 4 none mucous cells. Different letters indicate significance ($p \leq 0.05$).

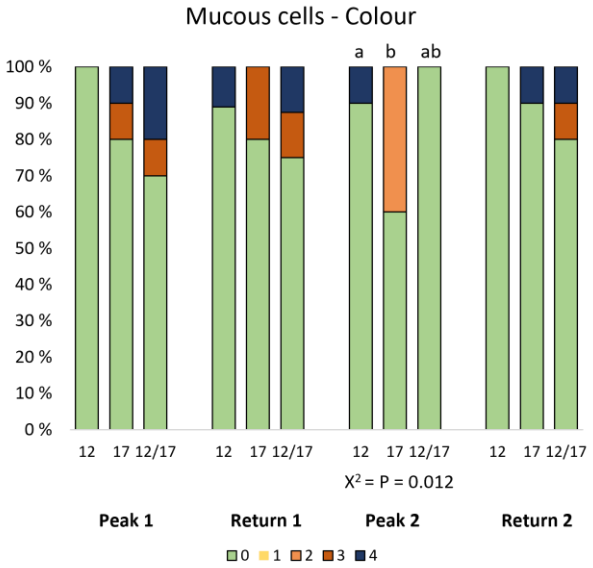

Figure 30. Histological analyses from criteria 3, mucous cells colour, in gill from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates dark blue only, score 1 pink, score 2 indicate a 50/50 mix of dark blue/pink, score 3 when 75% of mucous cells are dark blue and 25% pink, score 4 when 90% dark blue and 10% pink. Different letters indicate significance ($p \leq 0.05$).

Aneurisms

There was no significant presence of aneurisms in the gills in the different temperature groups, and the occurrence was overall low in all groups. Nevertheless, figure 31 shows a trend in increased presence of aneurism in the gills from FT compared to those from CT and HT.

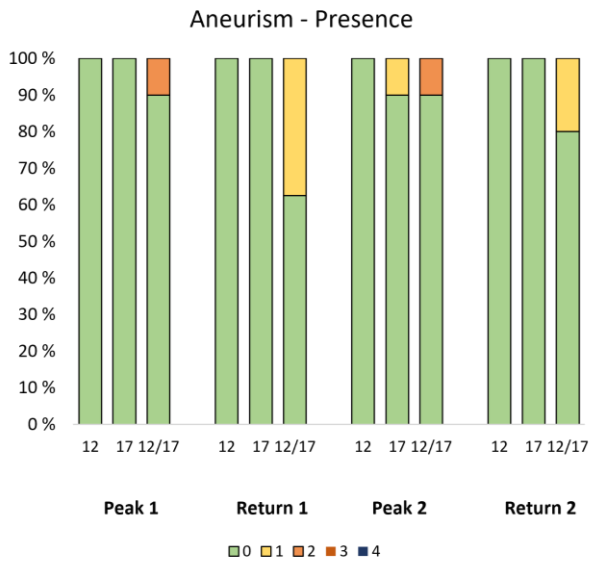

Figure 31. Histological analyses from criteria 4, aneurism, of gill from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates no presence, score 1 when few sporadic, score 2 indicate when evenly distributed – few, score 3 when evenly distributed – many and score 4 when extensive presence of aneurism. Different letters indicate significance ($p \leq 0.05$).

Lifting

Lifting was observed in a proportion of the gill samples and suspected to be associated with sampling-related handling. Lifting in the gills that were caused by the sampling were identified, and two figures were made to show the occurrence. Figure 32 includes samples with artefacts, while gill sections with clear lifting artefacts were excluded from figure 33. The level of lifting varies between the different temperature groups, and there are significant differences in both illustrations. In general, the percentage presence of lifting is highest in gills from FT compared with CT which essentially had a score of 0 (no lifting presence).

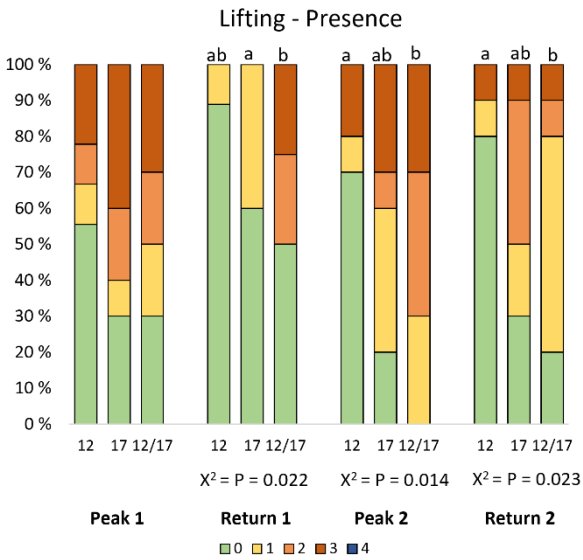

Figure 32. Histological analyses from criteria 5, lifting, in gill from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates no presence, score 1 when less than 10% has lifting, score 2 when moderate presence, score 3 when more than half of the gills has lifting and score 4 indicate that the whole gill has lifting present. Different letters indicate significance ($p \leq 0.05$).

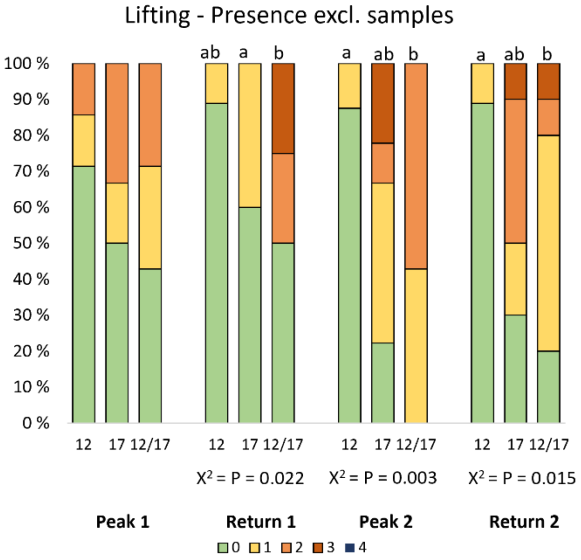

Figure 33. Histological analyses from criteria 5, lifting, in gill from post-smolt Atlantic salmon. Columns show distribution of score (0-4) in percentage. Score 0 indicates no presence, score 1 when less than 10% has lifting, score 2 when moderate presence, score 3 when more than half of the gills has lifting and score 4 indicate that the whole gill has lifting present. Different letters indicate significance ($p \leq 0.05$).

3.2.2 Digital scoring of gill-samples

Clubbing

Analyses of clubbing showed no significant differences between the temperature groups ($p = 0.897$). Nevertheless, figure 34 shows that there is an increase in lamellar clubbing for all temperature groups at Peak 2, and that the number of clubbing decreases until Return 2.

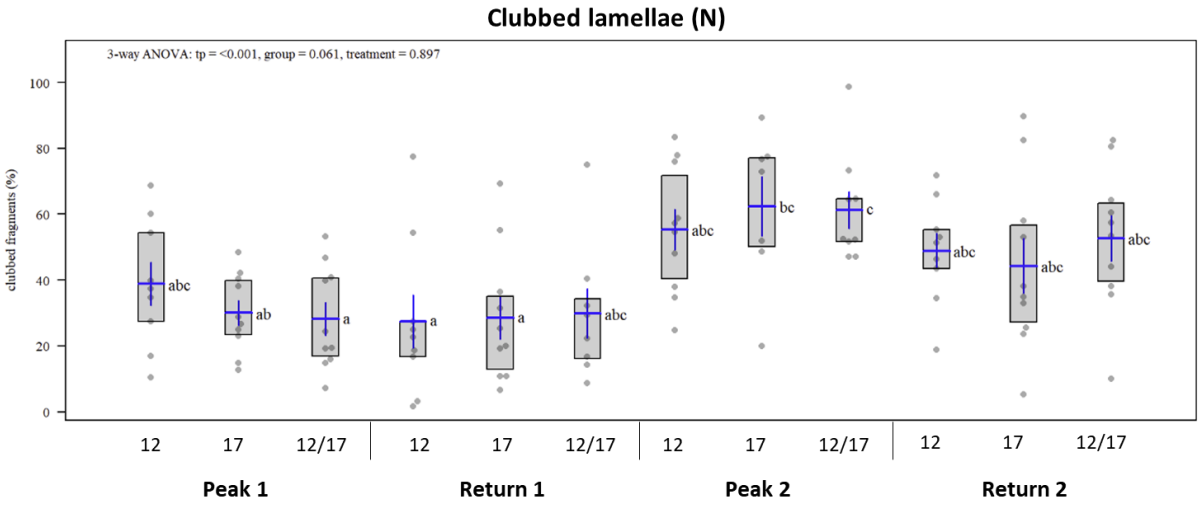

Figure 34. Aiforia® analysis data from tissue in gills of post-smolt Atlantic salmon. Box plots showing the development of measured number of clubbing in secondary lamellae. Statistical analysis, and presentation of data done by researchers at Nofima.

In Aiforia, secondary lamellae with identifiable clubbing were colored red, while the green areas indicate areas without clubbing (figure 35). The empty spaces between the secondary lamellae are not identified lamellae, where fusion has probably occurred. Fusion is a change that has not been classified in Aiforia yet.

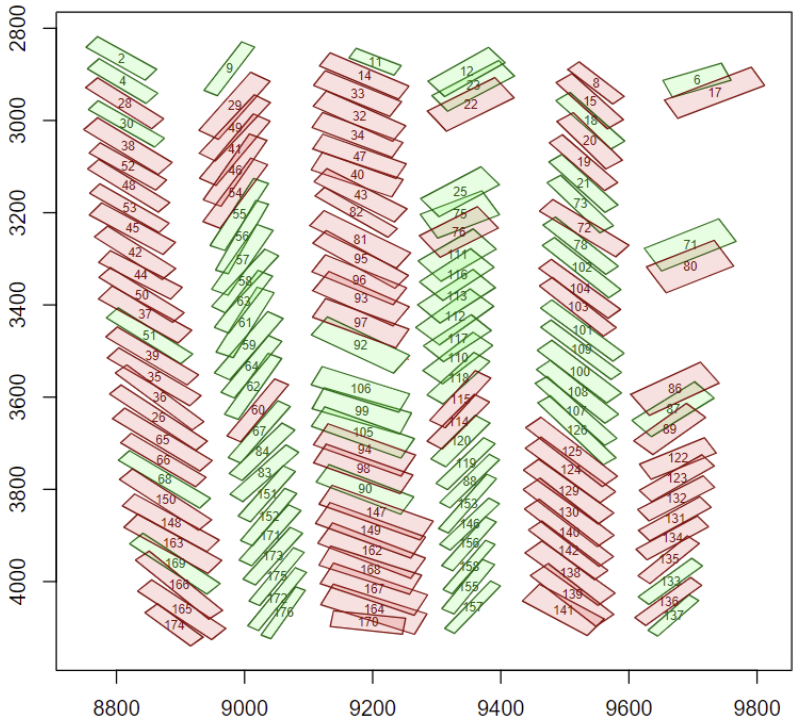

Figure 35. Illustration of clubbing detection of secondary lamellae by Aiforia. Red areas are lamellae with clubbing, and green areas indicate secondary lamellae without clubbing.

Mucous cells

The occurrence of mucous cells in the primary lamellae differed significantly between the different temperature groups (p = 0.009) (figure 36A). The same development is seen for the number of blue mucus cells in the primary lamellae between the temperature groups (p =0.01) (figure 36C). However, there was no significant difference in the percentage of mucous cells in the secondary lamellae (p =0.3) (figure 36B) or the number of blue mucous cells in the secondary lamellae (p =0.436) (figure 36D).

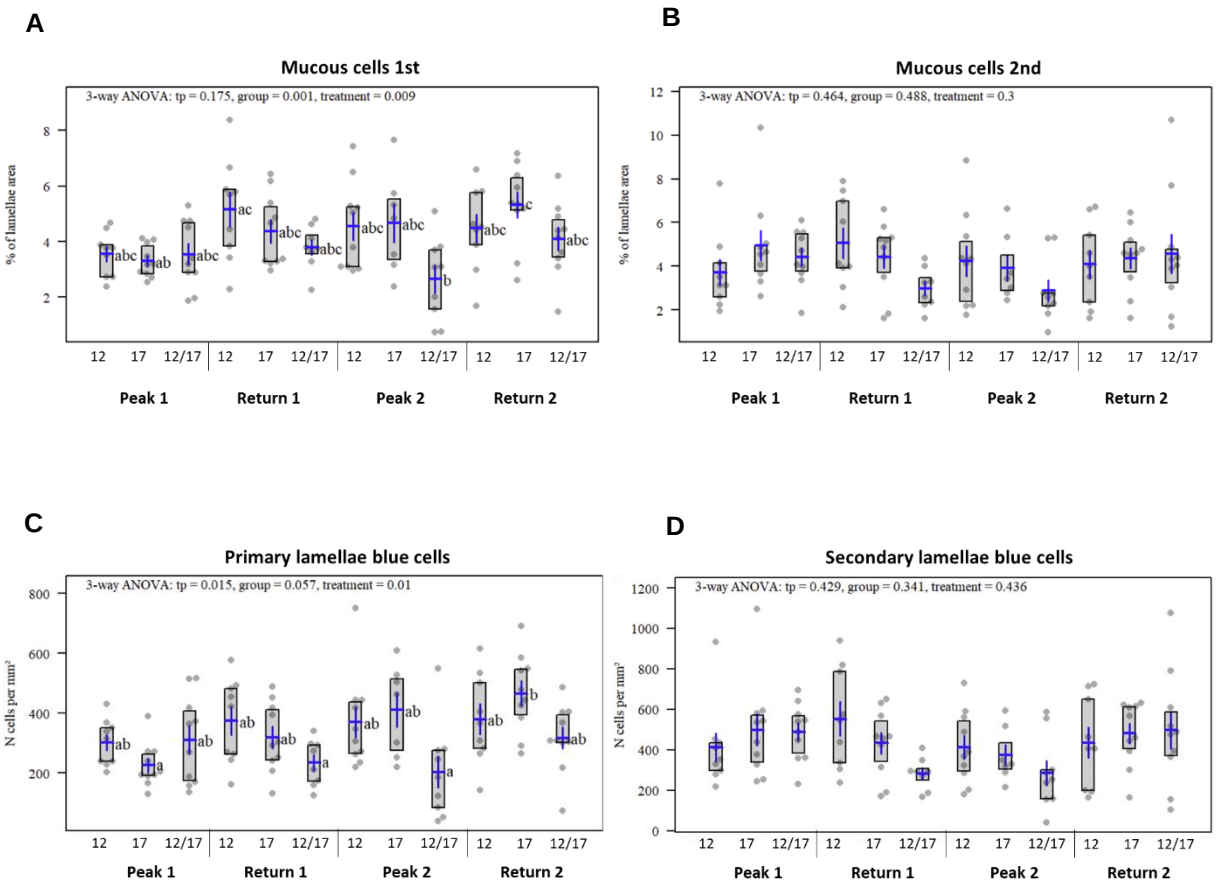

Figure 36. Aiforia® analysis data from tissue in gills of post-smolt Atlantic salmon. Box plots showing the development of measured number of clubbing in secondary lamellae. Statistical analysis, and presentation of data done by researchers at Nofima.

Aneurisms

No significant differences in the presence of aneurisms between the gills from CT, HT and FT were recorded in Aiforia (p =0.469), figure 37.

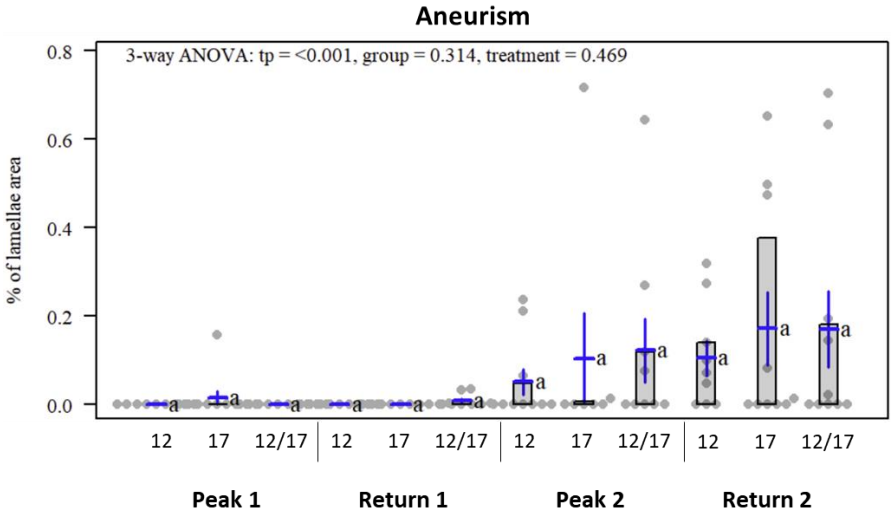

Figure 37. Aiforia® analysis data from tissue in gills of post-smolt Atlantic salmon. Box plots showing the development of aneurisms in the different temperature groups. Statistical analysis, and presentation of data done by researchers at Nofima.

Chloride cells

Chloride cells in the gills showed significant differences between the different temperature groups (p = <0.001). Gills from CT had a higher number of chloride cells than both HT and FT throughout the trial period (figure 38).

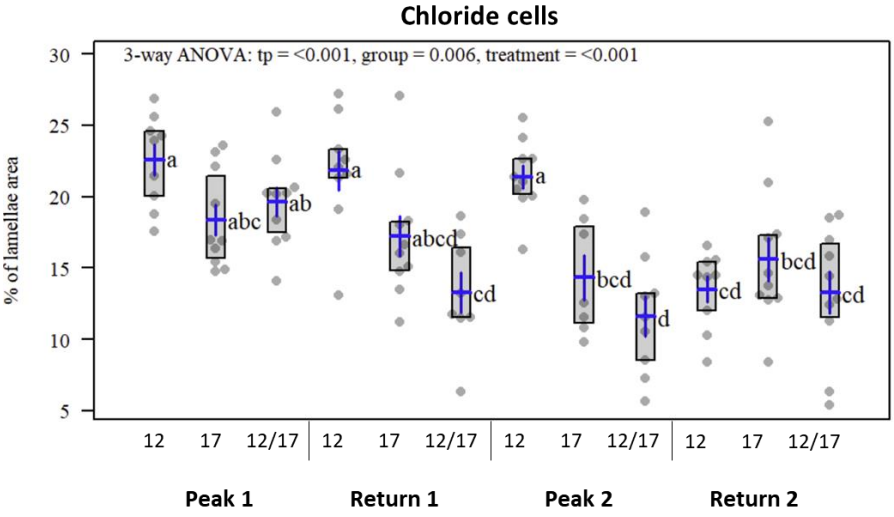

Figure 38. Aiforia® analysis data from tissue in gills of post-smolt Atlantic salmon. Box plots showing the development of measured number of chloride cells. Statistical analysis, and presentation of data done by researchers at Nofima.

Secondary lamellae length

The length (µm) of the secondary lamellae increased from Peak 1 to Return 1 for all temperature groups (figure 39). Although there are no significant changes between the different temperature groups (p =0.215), there is a clear change in Peak 2. In particular, gills exposed to HT had registered shorter secondary lamellae than those exposed to CT. At Return 2, the length of the secondary lamellae in all temperature groups is back to approximately the same length as at Return 1.

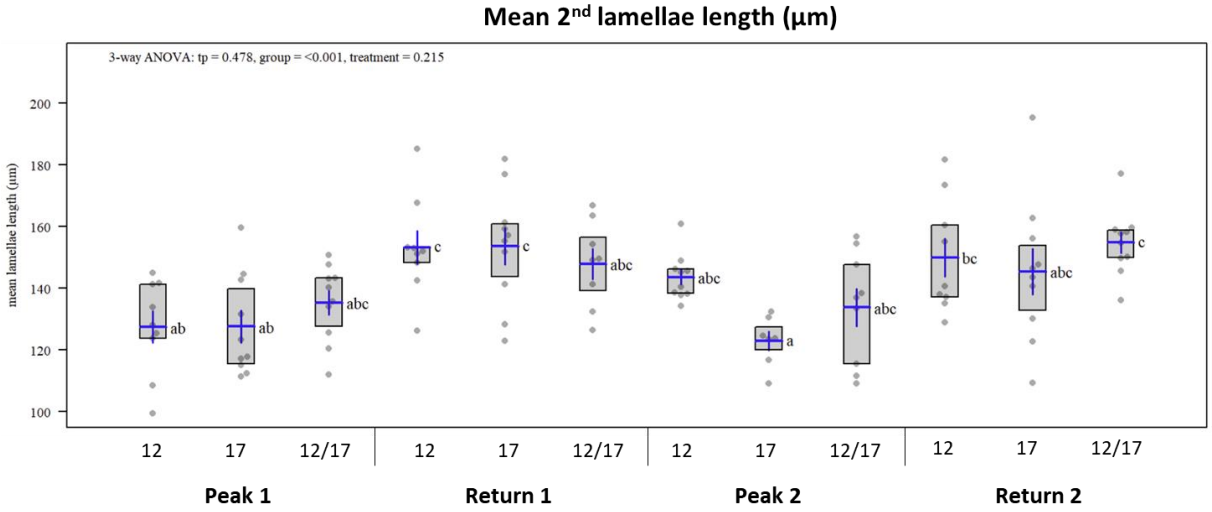

Figure 39. Aiforia® analysis data from tissue in the gills of post-smolt Atlantic salmon. Box plots showing the development of the secondary lamellae length (µm) in the different temperature groups. Statistical analysis, and presentation of data done by researchers at Nofima.

3.3 qPCR analyses

Expression of stress genes *hsp70, hsp90, Cu/zn sod* and *MnSOD* is shown in figure 40. No statistical differences were detected between the different temperature groups in either *hsp70* (figure 40A) or *hsp90* (figure 40B*)* expression. Even though, a higher relative expression of *hsp70* were detected in gills from CT in Return 2, compared with Return 1. The oxidative stress gene *Cu/zn sod* was significantly down regulated in FT, and up regulated in the other two temperatures in Peak 1 (figure 40C). After this, no significant changes were observed between the temperature groups. Significant differences were detected in *MnSOD* expression, which is also an oxidative stress gene, in the first two sampling time points (Peak 1 and Return 1) between the different temperatures (figure 40D). The *MnSOD* expression was down regulated in FT compared to the other groups in Peak 1, and up regulated in Return 1.

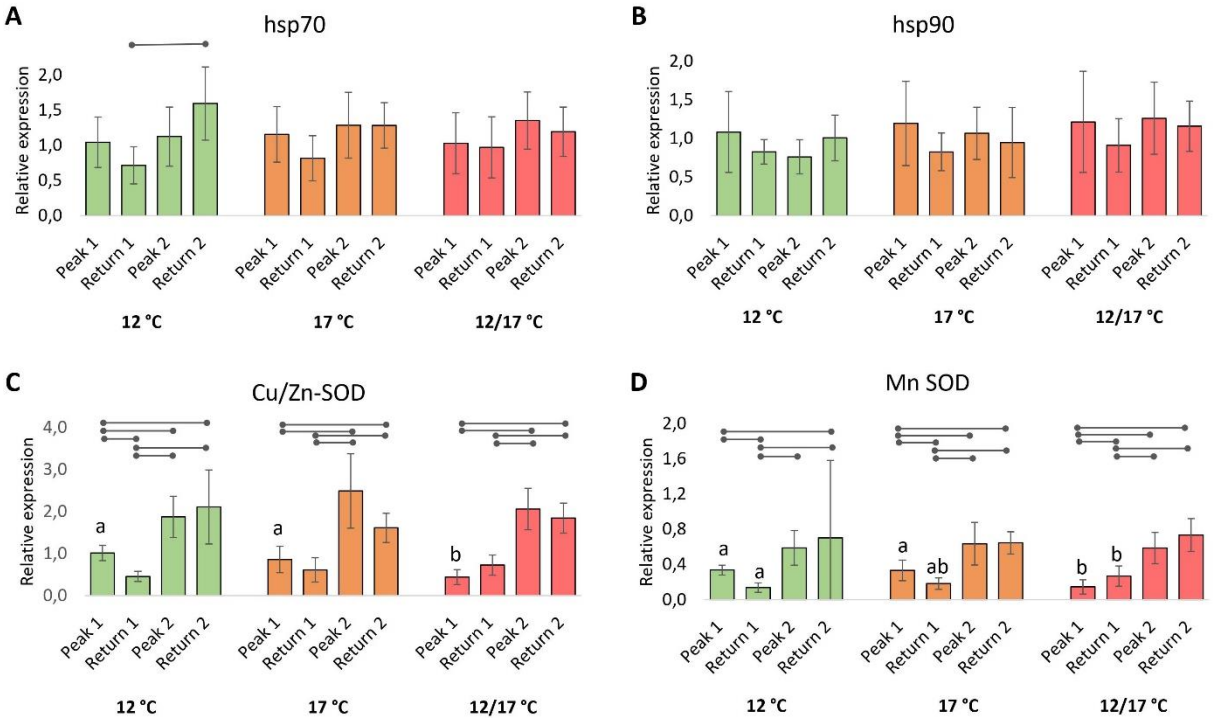

Figure 40. Gene expression level of stress genes hsp70, *hsp90, Cu/znSOD* and *MnSOD* in gill of Atlantic salmon. The y-axis shows relative expression of the different stress genes. Different letters indicate significant differences among the groups at a particular sampling point ($p \leq 0.05$). Significant differences within the temperature group are representing with lines ($p \leq 0.05$).

No significant variation in gene expression of the stress gene *glutathione peroxidase* (*gpx*) or the cytokine *interleukin-1β* (*il1β*) between the temperature groups was noticed (figure 41). Significant variation in sampling time point was found within the different temperature groups for *GPx*, but the same trend is seen in all groups (figure 41A). For *il1β*, there were only significant variations between sampling time points within HT and FT (figure 41B). In the CT, significant difference was recorded between Return 1 and Return 2.

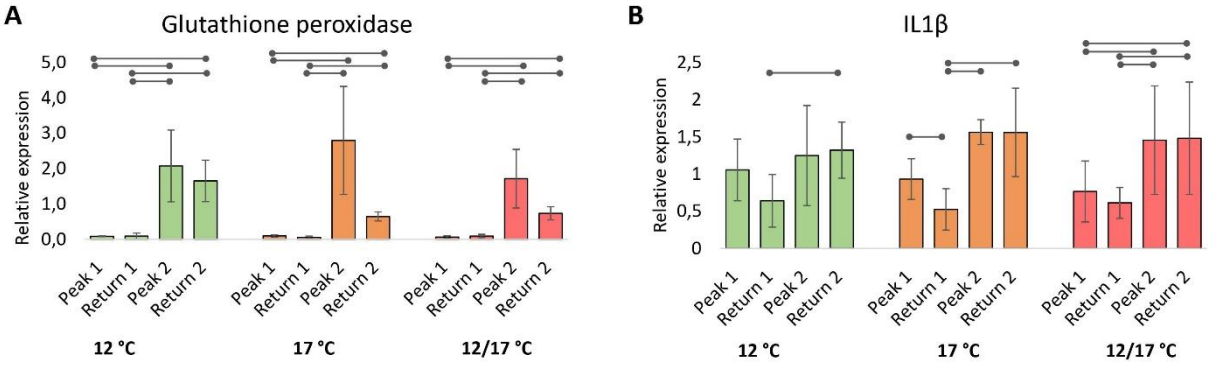

Figure 41. Gene expression level of stress gene *glutathione peroxidase* and cytokine *IL1β* in gill of Atlantic salmon. The y-axis shows relative expression of the different genes. Different letters indicate significant differences among the groups at a particular sampling point ($p \leq 0.05$). Significant differences within the temperature group are representing with lines ($p \leq 0.05$).

No significant differences were detected in the expression of the pain gene *trpv1* between the different temperature groups. In FT, significant differences were seen between the sampling time point Return 1 and Peak 2 /Return 2, with an upregulation of gene expression from Return 1 to the other two time points (figure 42A).

Expression of the apoptosis-related gene *casp3a* is not significantly different in CT, HT or FT. A downregulation of *casp3a* was statistically detected in Return 1 for CT and HT. No significant expression of *casp3a* was observed between time points in FT. Even though, figure 42B shows that FT have a relatively steady increase of *casp3a* expression between the different time points.

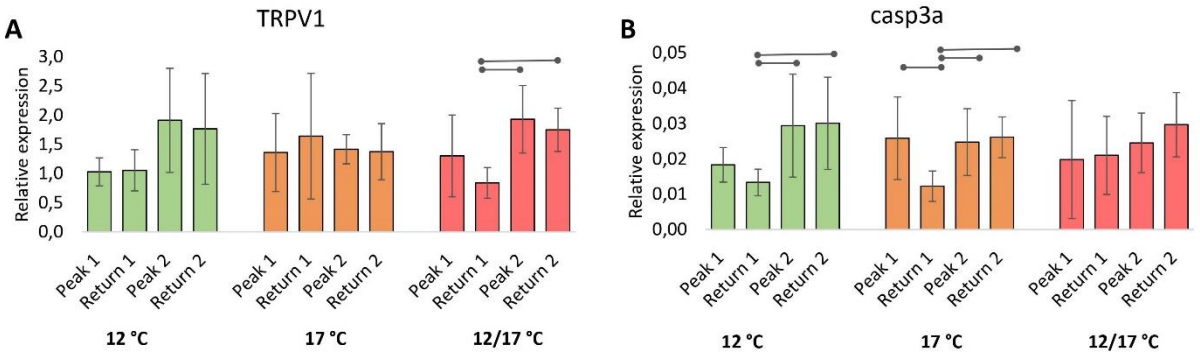

Figure 42. Gene expression level of pain detection gene *TRPV1* and the apoptosis gene casp3a in gill of Atlantic salmon. The y-axis shows relative expression of the different genes. Different letters indicate significant differences among the groups at a particular sampling point ($p \leq 0.05$). Significant differences within the temperature group are representing with lines ($p \leq 0.05$).

Relative expression of three structural genes, *claudin 10e*, *claudin 28a* and *collagen 1a* is shown in figure 43. A significant increase in *claudin 10e* expression was recorded between CT and FT in Return 1 (figure 43A). There was also a significant increase in expression within the temperature groups from heat wave 1 (Peak 1 and Return 1) to heat wave 2 (Peak 2 and Return 2). The same trend can be found for *claudin 28a*, except that there are no significant differences between the temperature groups, just within the temperature groups time points (figure 43B). There was also no significant difference in *collagen 1a* expression between the different temperature groups (figure 43C). However, a significantly elevated gene expression was found from Return 1 to Return 2 in CT. This increase is not seen in the other two treatments.

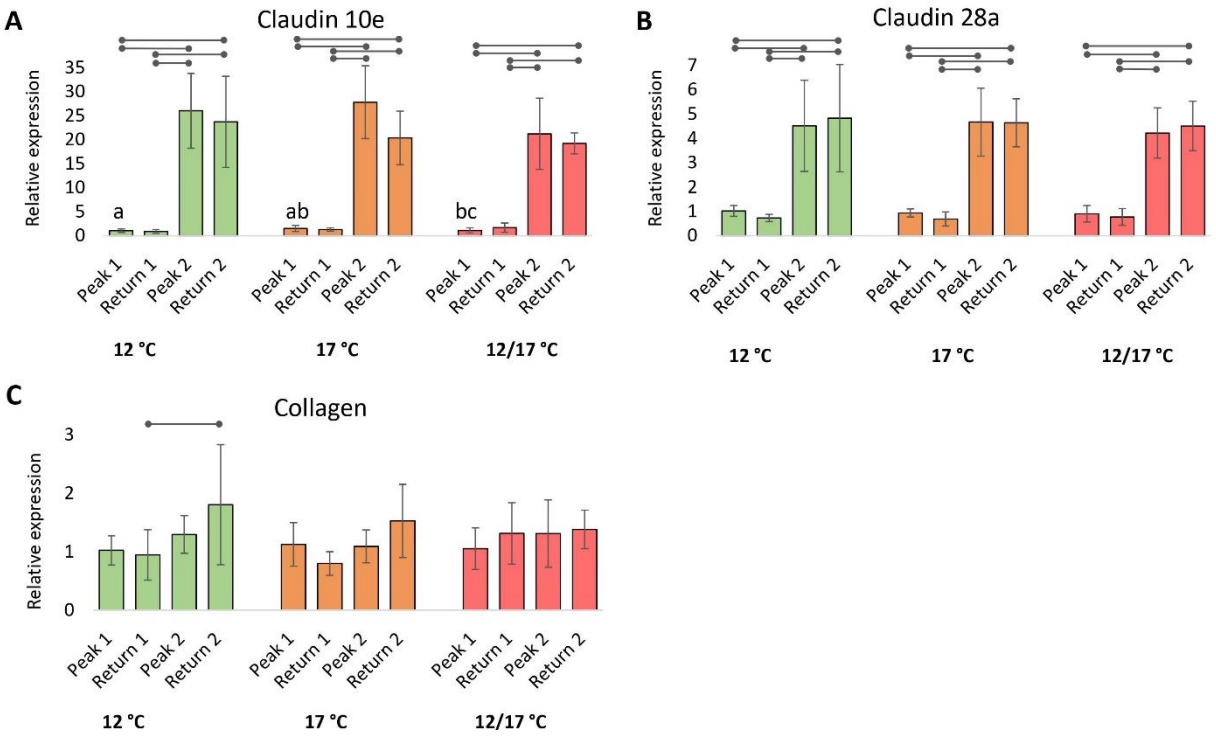

Figure 43. Gene expression level of different structural genes: *claudin10e, claudin28a* and *collagen 1a* in gill of Atlantic salmon. The y-axis shows relative expression of the different structural genes. Different letters indicate significant differences among the groups at a particular sampling point ($p \leq 0.05$). Significant differences within the temperature group are representing with lines ($p \leq 0.05$).

4. Discussion

In this work, histological changes and transcriptional responses in Atlantic salmon gills from constant high (17 °C) and fluctuating (12/17 °C) temperature conditions were compared to low temperatures (12 °C). Studying the impact of thermal changes may lead to a better understanding of future salmon farming under climate change, by documenting health and welfare challenges. High water temperature has shown to lead to reduced welfare, increased mortality and higher occurrence of various pathogenic organisms (Grefsrud et al., 2022). Gill problems are a considerable and growing problem for salmon in the marine phase, and have a major impact on the welfare of the fish (Grefsrud et al., 2022 ; Sommerset et al., 2023). These problems have shown to be particularly noticeable in late summer and autumn (Sommerset et al., 2023), when the water temperature is at its highest. In this study, it was shown that constant high and fluctuating temperature condition severely affect the gills. Morphological changes were higher in both constant high and fluctuating conditions. Gills from FT regime generally had several morphological changes compared to gills from HT, including the presence of fusion, clubbing and lifting. However, beyond the trial, an improvement in gill morphology was seen especially in the gills from fish in the HT compared to the gills from FT. These observations may indicate that fish at constant high temperature may adapt better to changes in temperature, while fluctuating temperature requires more of the fish to adapt.

4.1 Growth data

There were no general significant differences observed between the temperature groups. However, trends in weight gain were observed on the last sampling time point where the fish from FT had slightly higher mean weight compared to fish from CT and HT. Few studies are done on fluctuating temperatures, but compared to studies on higher temperatures, it is seen that growth of salmon is affected by temperature changes in the sea, where temperatures above 17 °C for a longer period have a negative effect (Gonçalves et al., 2006; Hevrøy et al., 2013). This trend has also been seen in previous sea temperature experiments, where it has been observed that feed intake increases with increasing temperature up to a threshold, before it then decreases (Hevrøy et al., 2013). It is therefore interesting to find that fluctuating temperatures also may lead to hampered growth. Growth can be used as a general indication of fish welfare and a way to see the temperature's effect on the fish. The "FishWell" project (Noble et al.,

2018) has done a review of the optimal water temperature range of salmon in different life stages, which shows that different researchers disagree about what is the optimal temperature for post-smolt (range 5 - 18 °C). In this thesis, the focus has not been on growth, but more used as a general sign of the fish's welfare. Still, it is interesting that no significant differences were detected between the treatment groups.

4.2 Histological changes in the gills caused by different temperatures

The temperature groups were compared according to the presence of various histological changes from the first sampling time point and until the end of the trial. By comparing gills from the different groups, one has the opportunity to investigate the degree of change the different temperatures cause. The distribution of the manual gill score showed that the changes were most pronounced in gills from FT and HT, compared to CT. The gills from CT had on average a lower score, indicating fewer morphological deviations. Even though the average manual gill score was low, between 0.5-1.5 out of 4, for all groups throughout the experiment, the changes can have important biological consequence for the fish, as some of the measures may have big consequences for the gill function.

4.2.1 Structural changes

Differences in both frequency and severity of lamellar fusions between the different temperature groups were observed. Overall, there was a higher severity and presence of fusions in gills from HT and FT compared to the CT in Return 1. However, after Return 1, the gills in the HT appeared recovered. Only the FT showed a higher appearance of fusions in Return 2. A change in environment will lead to new requirements for remodeling the salmon gills to accommodate the new respiratory and/or osmoregulatory changes in both a rapid and reversible way (Nilsson, 2007; Shwe et al., 2022; West et al., 2021). Research on the regeneration capacity of the gills of zebrafish (*Danio rerio*) has shown that the gills are rapidly remodeling, where half of resected tissue in gills is replaced within 40 days, and almost completely regenerated after 160 days (Mierzwa et al., 2020). This development is probably due to fact that different organ, including gills, contain mitotic cells with proliferating cell nuclear antigens (PCNA),

which is an essential component in the DNA metabolism and repair (Kelman, 1997). These mitotic cells are shown to increase in number in the secondary lamellae by gill irritations and contribute to regeneration (Mierzwa et al., 2020). Similar findings have been done in carp (*Carassius carassius*), where increased apoptosis, in combination with reduced cell proliferation was found in response to changes in oxygen accessibility in the water (Sollid et al., 2003). The reduction in lamellar fusions and the continuous present of fusions in gills from the FT may indicate that fish at constant high temperatures may adapt better to its environment.

An increase in the presence and size of clubbing was also observed with increasing water temperatures. Clubbing of secondary lamellae has become a change often seen in gills after handling operations (Grøntvedt et al., 2015). Studies done on gills from Atlantic salmon smolt in Tasmania showed clubbing, which eventually recovered without specific treatment (Clark et al., 1997). These gill changes were later found to most likely be caused by environmental factors, as no discoveries of pathogenic organisms were found. Previous research has shown that acidic water, elevated aluminum levels and/or cadmium, as well as increased levels of ammonia in connection with low pH, can cause lamellar clubbing (Brown et al., 1990; Klontz et al., 1985; Mueller et al., 1991) In these studies, water parameters were kept stable and monitored throughout the trial period, and water came from the same source to all tanks, so other causative factors to clubbing, such as increased levels of ammonia or low pH, is minimal. As mentioned earlier, higher water temperatures lead to reduced dissolved oxygen in the water, even if the water flow is kept the same in all tanks. In this study, oxygen was supplied to the tanks with high water temperatures, to ensure similar O_2 levels in all tanks and to ensure that temperature was the only changing factor.

The clubbing analysis in Aiforia did not confirm the manual scoring, and only identified significant differences between the time points. However, results indicated an increase in clubbing at Peak 2, with a reduction at Return 2, as also shown with the manual scoring. Going through the digital detection in the histological images, it may seem that the algorithm is still not entirely optimized, and more training of the algorithm is needed for this feature. In Aiforia, the width and length of the secondary lamellae are measured, where unusually wide lamella are registered as clubbing. As some sampling artefacts were observed in some of the samples, lamellae with lifting may have been identified as clubbing. In addition, the orientation of the

secondary lamellae may increase the width depending on the orientation of the organ, thus adding further problems for the algorithm to correctly identify clubbing. The comparison between manual and digital scoring done here shows how important verification of digital histopathology is, and that a critical evaluation of digitalized algorithms developed for biological assessment is especially important.

As aneurisms are one of the most common morphological changes observed in gills, it may be used as a quick indication of whether the gill is damaged. (Pharmaq, 2023). Research on gill in larvae and juveniles of the neotropical fish species (*Lophiosilurus alexandri*) and from coral reef fish (*Acanthochromis polyacanthus*) at elevated water temperatures (>30 °C) showed an increased incidence of gill damage, such as aneurisms, at increasing water temperatures (Rodgers et al., 2019; Takata et al., 2018). Even though there were no significant differences in aneurism presence between the temperature groups, both digital and manual analyses showed a trend of increased presence of aneurisms in gills from the FT. The manual score and digital score matched in the observations of Peak 2, where the FT had the highest incidence of aneurisms compared to the other two groups. Return 2 generally had few aneurisms according to the manual scoring. Aiforia recorded bleeding in all three temperature groups, with the highest presence in the gills from HT. Aiforia's results match the results from previous research, where an increased incidence of bleeding over time is seen in gills exposed to higher water temperatures (Grefsrud et al., 2022). The reason the aneurisms was not seen in the manual scoring may be that they were at an early stage with an incipient bleeding, which was not manually observed. There are several stages of aneurisms, and in some cases, the aneurism may have been recorded as clubbing (Dalum et al., 2018).

Lifting was observed in several samples from all groups, with higher appearance of lifting in gills from FT compared to the CT for the last two sampling time points (Peak 2 and Return 2). Lifting is often associated with artefacts, as it may occur post-mortem (Furnesvik et al., 2022). However, lifting and edema in the gills may be induced by e.g. thermal delousing using very high temperatures (Gismervik et al., 2019). Increased occurrence of lifting was seen in gills from both constant HT and FT, however, was only significant in the FT. It may be speculated

that lifting occurs more easily in already weakened tissue post-mortem, making the results interesting even if some of these changes occurred during sampling.

The secondary lamella length was measured in Aiforia and showed a tendency of increased lamellae length from Peak 1 to Return 1 in all temperature groups. The trend in increase of lamellae length is most likely caused by increased growth in fish (Don Stevens & Sutterlin, 1999), because as the fish grow, it is natural to think the lamellae also become longer. However, there was a trend for decreased lamellae length at Peak 2 for the gills from HT and FT. This gives rise to speculations if increased temperature may lead to shorter secondary lamellae in Atlantic salmon. Exposure of a number of coral reef species to high water temperatures, showed significant differences in the quantitative measurements made on the lamellae, where lamellar perimeter, cross-sectional area, base thickness and length of the lamellae were measured (Bowden et al., 2014). Moreover, populations of African cichlids and cyprinids adapted to hypoxic habitats have shown to have a greater respiratory surface area, such as increased gill filament length and lamellar surface area, compared to those living in well-oxygenated habitats (Chapman et al., 2000; Schaack & Chapman, 2003). These adaptations come from genetic evolution between populations and adaptive change during development, and can be assumed to lead to increased water inflow over the gills (Sollid & Nilsson, 2006). Overall, studies link lamellar length to advantages for fish in higher water temperatures as rising temperatures leads to less dissolved oxygen, but still increases metabolism, which will require more efficient oxygen uptake. As the results from this study show trends in shorter lamellae length in both the FT and the HT, increasing length may be associated with gill remodeling, as a response to a changing environment.

4.2.2 Changes at the cellular level

Analyzing the mucous cells showed a higher percentage of these cells in gills from HT and FT compared to gills from CT, which only had sporadic presence of mucous cells. Hypertrophy and hyperplasia of mucous cells are often used as an indication of gill disease (Ferguson et al., 1992), where excessive mucus production is often associated with increased gill irritation and an a reaction to unfavorable environmental conditions (Roberts & Smith, 2011). These statements are consistent with the results from this study, which shows that elevated and fluctuating water temperatures can lead to increased mucous cell production. A higher percentage of the mucous cells were located in the primary lamellae in the CT compared to the HT and the FT, which also had a larger number of mucous cells in the secondary lamellae. Previous study on the mucous cell position in the lamellae show that there are two types of mucous cell populations in the gills. Mucous cells in the primary lamellae are associated with the excretion of ions, minerals and metals, while those located in the secondary lamellae are more integrated with functions such as respiration and mucosal immunity (Haddeland et al., 2021). That more mucous cells were observed in the secondary lamellae in the gills in HT and the FT compared with the CT, could indicate higher gill irritations at higher temperatures.

The results from Aiforia showed a generally higher proportion of mucous cells in the primary and secondary lamellae in the CT and in the HT compared to the FT, in Return 1 and Peak 2. The reason for this is unknown but may be due to the ROI used in the digital detection. In the manual scoring, the score was set from the entire section and scores were given from a larger area than the digital scoring, where mainly three primary lamellae were analyzed. These differences must be taken into account in further digital analyses. The gills from HT had the highest number of mucous cells at Return 2, indicating increased gill irritation at 17 °C. As increased mucus production in the gills may lead to compromised gas exchange (Speare & Ferguson, 2006), this may cause further problems for fish in an environment with low content of dissolved oxygen, such as elevated sea water temperatures. Fish trials with multiple stressors, such as a combination of high water temperatures and lower oxygen levels, may increase our understanding of these responses in fish.

Dark blue mucous cells were dominating in the various temperature groups, but a higher occurrence of pink mucous cells was observed in gills from the HT, compared to the other two groups at Peak 2. In Aiforia, only significant differences were recorded in the number of blue

mucous cells in primary lamellae, where the gills from HT had highest incidence in Return 2. AB-PAS staining has been shown to stain the mucins blue at acidic pH, and pink at neutral pH (pH 7) (Smith et al., 2018). Why salmon produce different mucins are not fully understood, but recent studies suggest that the glycosylation pattern of the mucins changes as a response to e.g. thermal stress and pathogens (Linden et al., submitted 2023; Padra et al., 2019).

The Aiforia program has been trained to detect chloride cells digitally. This is an advantage of digital histopathology, as one does not have to count every cell manually, which is a laborious method. A higher number of chloride cells were counted in gills from CT compared to the gills from the HT and FT, which had a decrease in number of chloride cells in the first three sampling time points. Previous experiments on Atlantic salmon showed a distribution decrease in the number of chloride cells when exposed to AGD (Chang et al., 2019). The findings proved to be closely related to increasing levels of basal epithelial hyperplasia as a result of focal AGD lesions. The changes can be thought to lead to implications for the fish, as the chloride cells have important functions in ion and pH regulation, where a decrease in these cells may result in disfunctions (van der Heijden et al., 1999). Atlantic salmon has both a freshwater phase and a saltwater phase, and it is therefore difficult to conclude this changes result in implications. Nevertheless, there is still a noticeable change in temperature from 12-17 °C, so fluctuating stress may have an impact on the chloride cells functions without this being ascertained. Apoptosis of chloride cells has previously been described in, among several species, nearly hatched rainbow trout as a result of an adaptive response to various environmental changes (Røjø & Gonzaález, 1999). These findings show that diseases and environment can influence the presence of chloride cells in gills. Although the study looked at a different species at a different stage, the changes seen in this study may indicate that higher water temperatures and fluctuating temperatures might, like other environmental changes, lead to a decrease in chloride cell levels in salmon gills. What this will mean for the fish will be interesting to study further.

4.3 Gene expression

The changes in gills from fish exposed to different temperatures were also studied by the gene expression, including gene markers for stress genes (*hsp70, hsp90, Cu/zn sod, MnSOD* and *glutathione peroxidase*), immune genes (*cytokine il1β,* pain gene *trpv1*), apoptosis gene (*casp3a*) and structurally genes (*claudin10e*, *claudin28a* and *collagen 1a*). There were few significant differences in gene expression between the treatment groups, so the results from the qPCR analysis must be seen in correlation to the histopathological changes observed. In the following sections the various genes are discussed.

4.3.1 Stress genes

In a farmed context, several stressors often occur at the same time, and unlike wild fish, farmed fish will not be able to escape the stress factors. By exposing salmon to environmental stress, such as elevated water temperatures, increased expression of various stress genes is assumed. Stress can lead to altered cell response, and in worst case, damage to DNA, proteins and lipids (Irina, 2011). Heat Shock Proteins (HSPs) have been shown to be activated by stress at increased temperature and is part of the cells' natural defense mechanism in the protection of protein derivation, and can be expressed more under stress (Tavaria et al., 1996). Results from qPCR using primers for *hsp70* and *hsp90* showed no significant differences in expression between the temperature groups. This was unexpected, especially considering that the *hsp70* showed a significant increase in expression from Return 1 to Return 2 in the CT, and not in the other temperature groups. Nevertheless, it is known that HSPs are continuously expressed in the cells, even in normal condition, as they also have a cellular function (Tavaria et al., 1996), high expression of HSP thus do not necessarily indicate increased stress response. Possibly an unchanged HSP expression can also mean that fish may be able to tolerate these conditions as it was optimal environment. However, this cannot be concluded by looking at the expression of just these genes. The *hsp90* can be activated quickly in response to acute stress, but also by prolonged stress exposure (Grefsrud et al., 2022). Although there were no significant differences between the groups, there is a small trend in higher expressions of *hsp90* in Peak 2 in gills from FT and HT compared to CT, which may be stress related.

Oxidative stress is a result in overproduction of Reactive Oxygen Species (ROS) that the antioxidants are unable to remove in a response to a reduction in oxygen accessibility. Antioxidants will try to prevent or repair the effects of oxidative stress, which can cause DNA damage. The antioxidants can be enzymatic as superoxide dismutase (SOD) and Glutathione Peroxidase (GPx) (Birnie-Gauvin et al., 2017; Valko et al., 2007). *Cu/zn sod*, *Mn SOD* and *GPx* are antioxidants tested in this thesis and will often be upregulated under stress (Hayes et al., 2005; Iwama et al., 2004). There were significant differences in relative expression in Peak 1 for *Cu/zn sod*, with a lower expression in gills from the FT. This trend is also seen for *Mn SOD*, where the FT has a down-regulation of relative expression in Peak 1 compared to the other two temperature groups. Downregulation could be a sign of a weakening of the inherent stress response before the fish eventually recover through time. No significant differences in gene expression of *GPx* between treatments groups, but the results showed an increase in *GPx* activity from HW1 to HW2 that may result from longer time spent in small tanks, sampling and other stressor related to general rearing conditions. In response to heat stress, activity was increased at Peak 2 in the HT and the FT compered to Return 2. *GPx* is previously mentioned an important antioxidant enzyme, that participate in cellular defense through the enzymatic reduction of reactive cellular hydroperoxides (Imai & Nakagawa, 2003; Kühn & Borchert, 2002; Wang et al., 2012). An increased differences between Peak and Return in the HT and the FT may indicate higher stress levels in these groups.

Study done on the liver of Atlantic salmon exposed to stress related to heat was shown to downregulate several transcripts encoding proteins involved in protection against oxidative stress, including *Cu/zn sod*, *Mn SOD* and *GPx* (Olsvik et al., 2013). *Cu/zn sod* showed a decreasing expression with increasing temperature, and was less expressed in livers kept at 17 and 19 °C compared to the control fish at 13 °C. *Mn SOD* was lower expressed in fish at 19 °C, compared to 13 °C. This observation was also made for expression of *GPx* (Olsvik et al., 2013). This can be caused to reduced antioxidant production as a result of reduced total metabolism at higher water temperatures. In general, elevated temperature actually leads to increased oxygen consumption and ROS production, and thus also increased oxidative stress in the fish, not the opposite (Heise et al., 2006). In this study, water circulation was maintained in all temperature groups, which does not lead to a change in the dissolved oxygen level in the tanks. Nevertheless, oxidative stress can be affected by starvation. Previous experiments on rainbow trout and brown trout (*Salmo trutta*) showed different enzymatic activity of the antioxidant genes during

starvation, which means that the effect of oxidative stress due to lack of nutrient absorption is difficult to determine in salmonids (Bayir et al., 2011; Furné et al., 2009).

4.3.2 Other temperature responses

In this thesis there was no significant differences in interleukin 1 beta (*il1β*) expression between the treatment groups. However, an increase of *il1β* expression within the HT from Return 1 to Peak 2/ Return 2, and from HWI to HW2 in the FT was observed. There was almost a steady relative expression of *il1β* in the control group. *il1β* is a pro-inflammatory cytokine secreted from innate immune cells such as monocytes and macrophages in immune response (Lopez-Castejon & Brough, 2011), as a host defense response to infection and damage (Dinarello, 1996). With this information, increased expression of *il1β* in HT and in the FT could mean increased cell damage during the trial period. This is supported by the observations made on histological sections of gills at these temperatures in this study, but also by previous research (Gismervik et al., 2019; Rodgers et al., 2019; Sollid & Nilsson, 2006).

Casp3a is usually activated in connection with a programmed cell death process called apoptosis. This activation can be caused by a number of signals, which include cell stress, DNA damage and cytokines, to contribute to apoptotic cell death by breaking down cellular structure and loss of function (Walsh et al., 2008). Investigations of transcription changes of various genes in brain samples from rainbow trout exposed to different water temperatures (10/15/20/25 °C), showed that stress caused by high temperature led to physiological changes in the fish brain. This by leading to reduced expression of *SOD* and *GPx*, and an increase in mRNA expression of *casp3, hsp70* and *hsp90* (Topal et al., 2021). No significant difference in expression was observed between the temperature groups in this experiment. A reduction of *casp3a* expression is seen for Return 1 in both the CT and the HT, while it has no change in expression from Peak 1 to Return 2 for the FT. There is a general low expression of *casp3a* in the gills (<0.05), which may indicate low activation of *casp3a* in this study. This observation does not correspond to previous research at high water temperature in the brain of rainbow trout, but may probably be due to a short exposure time, which could have been studied more carefully with a longer trial period.

There were no significant differences in *trpv1* expression in gills between CT, HT, or the FT. *trpv1 is* involved in pain perception and thermoregulation. This function is important in detecting hot temperatures and avoiding damage (Caterina et al., 1999). An increased expression of *trpv1* can thus mean an increased response to temperature change from 12 °C to 17 °C in the gills from FT. Previous studies on zebrafish show that *trpv1* is activated by environmental warm temperatures and is required for normal behavioral responses to heat (Gau et al., 2013). In zebrafish, *trpv1* functions as a molecular sensor for environmental heat (>25 °C) and is used as a direct sense of environmental heat. An expression of *trpv1* will therefore not necessarily indicate pain perception at higher temperatures, but a presence in the cell as a necessity to be able to detect abnormal environmental heat and prepare the fish for heat-induced responses. The activation of *trpv1* will probably vary greatly depending on the species' preferred water temperature.

4.3.3 Structural genes

In this study, the focus was directed at three structural genes, *claudin 10e, claudin 28a* and *collagen 1a*. Structural genes are genes that code for proteins that cells need for structure or function. No significant difference in expression of *claudin 10e, claudin 28a* and *collagen 1a* was observed between the temperature groups, only in Peak 1 for *claudin 10e*. There was an increase in the expression of *claudin 10e* and *claudin 28a* for all three temperature groups from Peak 1/Return 1 to Peak2/Return 2. This change in expression must probably have something to do with the development of the gills, as no differences are seen between the treatment groups.

Claudin genes can have different specific functional roles at different stages in the fish, which means that these genes are complex to study. Epithelial cells are tightly packed through e.g. tight junctions, with claudins being one of the most important proteins (Günzel & Fromm, 2012). Previous studies of *claudin 10e* have shown to be stimulated by smoltification before being reactivated by salt transfer (Tipsmark et al., 2008) and in response to high fish density (Sveen et al., 2016). This does not correspond with the results from this research, as one would then have expected a decrease in expression from Peak 1 to Return 2, not an increase in expression, as the fish in this trial have been fully smoltified and acclimatized in seawater. Perhaps the claudin genes are more expressed during the trial period to stabilize the gills and

strengthen the barriers. Possibly, increased biomass in the tanks can also cause this development in gene expression, as claudins have shown to up-regulate in relation to stress by increased biomass (Sveen et al., 2016).

No significant differences in *collagen 1a* expression was observed between the CT, the HT and the FT during the experimental period. However, a significant difference in relative expression for *collagen 1a* from Return 1 to Return 2 was observed in the CT. *Collagen 1a* is an important structural component of connective tissue and is an important component of gill cartilage. In channel catfish *Ictalurus punctatus* infected with Henneguya spp., destruction of the collagen in the gill cartilage was observed, which caused weakness and breakage of the gill filaments. These observations were made before the parasite was detected, which leads to assumptions that the cartilage breakdown occurred in the early phase of the development of inflammation in the gill (Lovy et al., 2011). In addition, it has been seen that stimulated neutrophils in catfish have been able to break down type 1 collagen (Noya et al., 1999). These cells are common defense cells in the immune system of fish (Ainsworth, 1992). In contrast to the CT, the HT or the FT do not have a significant increase in expression at the time point. The observation may indicate that higher and fluctuating temperatures lead to a stop in collagen production. Another study on collagen deposition in zebrafish showed to be enhanced in lower temperatures, and downregulated when the temperature increased (Lin et al., 2022). This agrees with the results presented in this experiment. Taken together, the development of the FT and the HT may indicate that elevated as well as fluctuating temperatures help to cause responses that contribute to the breakdown of the gill filament cartilage. Fish have shown to invest in growth and production of gonads by excess energy intake (Jobling, 1992). At high water temperatures or other unfavorable conditions, it is possible that the energy is instead used to maintain vital functions and stress response, while growth is reduced, as previously shown for other species, like cod (Björnsson et al., 2001).

4.4 General implications of compromised gill health

Histological examinations of gills exposed to elevated temperatures have shown to lead to reduced gill health. In this thesis, the effect of secondary stressors as a consequence of higher water temperatures has not been considered. Instead, two types of temperature stress were analyzed, continuous high and fluctuating conditions. In aquaculture, secondary stressors arising from altered environmental conditions, are as important as the direct effect of climate change. For example, the emergence of new pathogens, as well as increased spread and virulence of existing pathogens, are expected results of changed water temperature (Klinger et al., 2017). Fish diseases often lead to reduced fish welfare and may have major consequences on the direct impact on fish, which in the worst case can lead to mortalities. In addition, the diseases are bottlenecks for the aquaculture industry, where outbreaks can lead to large financial costs due to increased mortality or requirements for slaughter. It is therefore important to understand how fluctuating environmental conditions impact the general health and welfare of the farmed animals, so that especially vulnerable periods can be followed more closely.

Paramoeba perurans (synonym *Neoparamoeba perurans*), an amoeba parasite causing AGD, is seen as one of the main causes of pathological changes in the gills. Previously work has shown an association between temperature and variation in AGD severity in Atlantic salmon (Benedicenti et al., 2019). The results of the study showed that the occurrence of histopathological changes of amoeba increased and became stronger in salmon exposed to higher temperatures (15 °C), compared to the control group in the lower temperature group (10 °C) (Benedicenti et al., 2019). The infection pressure of salmon lice (*Lepeophtheirus salmonis*) is also temperature dependent, where increased water temperatures lead to increased reproduction and faster development of the lice (Dalvin et al., 2020; Sandvik et al., 2021). Salmon lice in itself is a major fish welfare problem, and with ongoing climate changes the number of treatments that remove the lice will increase. A combination of increased temperatures and more delousing operations can be too much for the fish (Burke et al., 2020). The probability that salmon will have increased problems related to gill diseases such as AGD and salmon lice in the future is thus expected.

High number of disease outbreaks during the sea phase is an ongoing problem, where outbreaks is often seen in the post-smolt stage (Sommerset et al., 2023). The reason for this is not entirely clear. However, various studies have found that there is a reduction in the IgM level, the number

of leukocytes and a change in leukocyte distribution in salmon during smoltification, indicating a significant weakening of immunity and an increased risk of infections (Jensen et al., 2019; Melingen et al., 1995; Pettersen et al., 2003). Trials with virus exposure during the smoltification process showed significantly higher accumulated mortality in the fish group that was ready for seawater transfer compared to the salmon group that was early in the smoltification process (Jensen et al., 2019). Smolt are exposed to different water temperatures depending on the time of year they are transferred to sea, which is usually in spring or autumn (Noble et al., 2018). Sea transfer in autumn coincides with a period of large reservoirs of larvae and salmon lice, but is also a period of lower temperatures that increase exposure to wound damage (Stefansson et al., 2005). Smolt transferred to sea in the spring are exposed to rising water temperatures, which have been seen through this study increases the risk of gill damage. When the gill barrier is damaged it is more susceptible to infections by algae, jellyfish and pathogenic organisms that thrive in higher water temperatures (Sommerset et al., 2023).

In conventional salmon farming, the fish are subjected to a lot of stress linked to the various procedures during the production cycle. Handling is a particularly stressful experience for the fish and is carried out in many phases throughout the production, like transport from hatchery to sea cages, sorting, overlining, weighing, delousing, salmon lice counting, sampling and removal of weak individuals. In addition, the fish is handled extensively when e.g., transported to the slaughter house. Fish that experience chronic stress, i.e. long-term stress, will have a reduced immune system, health and growth, which will make the fish more susceptible to invading pathogens that enter via injuries and wounds, possibly causing long-term suffering (Grefsrud et al., 2022). These factors together with a reduced gill health, as a result of high water temperature, will probably have compounding effects.

5. Conclusion

Elevated and fluctuating temperature conditions have shown to have an effect on gill health in Atlantic salmon. The histological changes in the gills from the HT were related to the first time exposed to 17 °C, which also applies to the FT, where most changes were seen after the first HW. However, gills from FT generally had several morphological changes compared to gills from HT, indicating that Atlantic salmon gills have difficulties adapting to fluctuating temperatures, and to recover. An increased gene response was generally not seen in the gills exposed to increased and fluctuating temperatures, but some genes associated with oxidative stress showed more expression in the gills from FT in the first HW, but not in the second HW. These changes may be a response to treatment, where the gills are to some extent able to adapt and recover. Nevertheless, in this study only one organ has been looked at, and perhaps the changes have greater significance in other parts of the fish. The effect of fluctuating temperatures on fish health should be further studied to fully understand the fish response to increased water temperature.

Findings indicate that the effects of climate-related temperature stress induce changes in the gills that may further make the fish more vulnerable to other stressors, such as other climate related stressors (e.g., oxygen depletion and reduced pH) and production related stress (e.g. pathogens, treatments and handling). The combination of factors may be compounding with elevated sea temperatures.

6. Future perspectives

The histological changes in the gills after exposure to different water temperatures, show that the environment surrounding the fish affects the health of the gills. There is still a need for understanding details of how fish respond to sub-optimal conditions rising from climate change. These changes can have a major impact on the fish's health, robustness, and general survival in the sea. Gills can give an indication that something is wrong, where further research on this topic can help to influence decisions that salmon farmers make for operations, such as de-lice and transport, as sick fish can have a reduced tolerance for handling and stress. With increasing seawater temperatures, more handling related to treatments during disease and parasite outbreaks is expected (Falconer et al., 2022). Perhaps one can make farmers to become extra observant of fish and gill quality when the water temperature is 17-18 °C. Being able to make these recommendations shows the importance of studying climate change and designing experiments to learn more about the fish health problems. For further experiments, it would be interesting to see how the gills had been affected by different ramp rates in temperature increase.

A limitation of this study is that only a small proportion of genes have been looked at compared to all genes transcribed in the gills, and thus just show a minor part of the transcriptional effects of temperature increase on the gills. Several different methods, experiments, and factors should therefore be carried out in order to gain a greater understanding of climate change.

There are several newspapers and magazines who writes about the problem of heat waves, where you can still find more information about this in the news than in the literature. The reason for this is that research and publications on this topic are lagging behind. Nevertheless, in the future one can expect increased publications on this topic, as many researchers, farmers and other stakeholders find this to be an important topic.

7. References

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