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Morphological and gene expressional effects of a 30 second warmwater treatment on Atlantic salmon (*Salmo salar* L.).

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Forord

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Abstract

The economic growth of the Atlantic salmon fish farming industry in Norway has lessened the last few of years. One of the reasons for this is the ectoparasite *Lepeophtheirus salmonis*, commonly known as the salmon louse. It feeds of the mucus layer and skin of the Atlantic salmon causing losses in quality of the farmed salmon. The louse is also a problem for the population of wild salmon passing through fjords near fish farms on their migration and spawning runs. Regulatory measurements were put in place to regulate the number of lice in fish farms. These measurements involved the use of chemotherapeutants the louse developed resistance towards over time. To circumvent the resistance situation, non-medicinal methods (NMMs) were developed for delousing. These include fresh- or warm water treatments, brushing, water currents and more. The Thermolicer is one of these NMMs and use water holding between 28-34 °C to delouse Atlantic salmon in fish farms. The welfare and effect documentation for the Thermolicer from 2015 found acute gill bleedings in fish from the tested fish farms. In 2020 the Thermolicer is the most commonly used NMM for delousing operations of Atlantic salmon.

In this experiment we examined the effect of treating Atlantic salmon with water holding 34 °C for 30 seconds. In this experiment the fish was sampled day 1 and day 21 post-treatment. The warm-water treatments were divided into in two sections with two groups in each. In both sections there was one group treated with warm water (34 °C) and one treated with cold water (8 °C) for comparison. Section one went through one cold-water treatment before being divided into warm- and cold-water groups and let rest for three weeks before sampling. Section two went through two cold-water treatments before division into warm and cold-water groups. Section two was treated, euthanised and sampled without rest. In the next experiment we sampled Atlantic salmon from two different full-scale Thermolicer treatments at two Norwegian fish farms. In this experiment the fish was sampled at day 0 and day 10. The field Thermolicer treatment used 29 °C with a temperature difference (delta t) of 23,5 °C. For both experiments the fish was welfare scored and the organ samples were examined histologically and through qPCR-analysis.

Our results showed no significant change in the scale loss and skin bleeding scores between fish treated with 34 °C and 8 °C. Scale loss was significantly increased at day 0 following the full-scale treatment. There was no significant difference in skin bleeding at day 0 following the full-scale treatment. There was no significant difference in thymus and pseudobranch between 34 °C and field treated, but a significant difference in affected tissue between gills from day 1 8 °C and 34°C (hypertrophy, lifting, or bleeding in histological sections). In addition, we investigated the number and acidity of mucus cell acidity in histological sections of skin and gill from both 8 °C, 34 °C and full-scale treatment. There was no significant difference between the 8 °C, 34 °C and field treated populations in acidity, but a lower overall number of mucus cells was recorded in the skin and gill sections from the field treated population. Head kidneys and gills were analysed expression of the following genes; interleukin 1β, interleukin 6, tumour necrosis factor α, heat shock protein 70 and complement factor c3. There were no significant differences in expression of the genes between the different treatment groups.

In conclusion, our results demonstrated small differences between fish treated at 34 °C and full-scale Thermolicer treated. However, there was a difference in treatment temperature of 34 °C and the Thermolicer treatment at 29 °C. This may suggest that Thermolicer treatments are not as harmful and may be a good method for delousing Atlantic salmon in fish farms.

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1. Introduction

1.1 Introduction to Atlantic salmon aquaculture in Norway

The Norwegian aquaculture industry of Atlantic salmon (*Salmo salar* L.) started at the end of the 1960s/early 1970s when the first smolt was transferred to sea and placed in an octagon shaped sea cage by Ove and Sivert Grøntvedt [1]. The first farmed Atlantic salmon was slaughtered in 1971. This was the pioneering work that started, what is today, a multi-billion export industry. The aquaculture industry today is mainly based on the rearing and export of Atlantic salmon, but also Rainbow trout (*Oncorhynchus mykiss* Walbaum) is farmed for export. In 2019 there were over 288 million individuals of Atlantic salmon in sea cages along the coast of Norway [2] equalling a slaughter volume of 1,28 million metric tons [3]. 1,1 million metric tons of slaughtered salmon was exported in 2019 at a value of 72,5 billion NOK [4].

The seafood report from the Ministry of Fisheries and Coastal affairs in 2013 refers to a list of reports, premises and goals for the future of aquaculture in Norway. One of the reports estimates the potential value of the aquaculture industry to be worth 500 billion NOK by 2050, five times the industry's worth today [5]. For this to be achieved and sustained there must be a political drive for sustainability in the regulation of the aquaculture industry [6]. Sustainable aquaculture is to be achieved through a knowledge based production with a focus on best practice and a predictable regulatory system [7]. There are several factors challenging the sustainability of the Atlantic salmon aquaculture industry today; amoebic gill disease, pancreas disease, mortality of cleaner fish, infections of salmon louse and the spread of ILA to mention a few [8]. Of these, the infection of salmon louse on Atlantic salmon in sea cages and potential spread to wild salmon populations is amongst the most challenging to handle [8].

1.2 The Atlantic salmon (*Salmo salar* L.)

The Atlantic salmon is an anadromous teleost fish belonging to the salmonid family whose life cycle begins and ends in the freshwater rivers connected to the northern part of the Atlantic ocean [9]. In the wild, eggs are laid in the gravel of the riverbed in the autumn by a mature female and are fertilised by a mature male's milt. After hatching they are known as alevins, small larvae of 15-25 mm with an attached yolk sac which sustains them through the first few weeks of their life [10]. It remains in the river as it goes from alevin to fry and end up as parr the following autumn at the size of 4-6 cm [10]. During the winter they enter a docile state of being in while lying in shelter of the rocks along the riverbed. In the spring the parr quickly gain weight and by the autumn of its second year it has darkly coloured bars (parr marks) along its green and silver tinted body [10]. The young parr usually spends between 2-7 years as a parr before going through a process called smoltification [11] depending on its nutritional status and environmental triggers [12]. Smoltification is a series of morphological, physiological and behavioural changes the parr undergoes to become adapted freshwater (hypoosmotic environment) to a life in seawater (hyperosmotic environment) [13].

The young salmon is now called a smolt, is around 10 cm long and 15-20 gram and the parr marks are gone [14]. Instead, the smolt is clad with silvery scales along its sides, a pale belly and a dark back, helping to conceal itself in the open water environment [12]. As these changes start to occur the smolt starts swimming downstream towards the sea where it will spend the next 1-5 years feeding and growing in size [9]. While swimming through the fjords and coastal waters the smolt is swimming at a depth of between 1-3 meters during the day, moving closer to the surface at less than 0,5 meters at night, depending on light conditions [15]. This migration is known to increase the fecundity, meaning increased capability for creating offspring, of mature salmon females [16]. The migration is an example of risk versus rewards, the potential growth and chance to procreate after a successful migration versus the high mortality of the migrating smolts [16]. According to the International council for the exploration of the sea (ICES), the mortality is between 90 and 99 % of the migrating smolt [17].

The mortality of Atlantic salmon smolt is caused by several factors, among these are: predatory birds, fish and mammals [18] along with diseases [19] and parasites [2]. The main portion of

the mortality is believed to occur in the first months after leaving the river [18] based on the assumption that there are more predators in the coastal areas than in the open ocean [9].

After spending 1-5 years at sea, the remaining salmon population swim towards coastal water trying to navigate itself back to its natal river [12]. The size of a mature Atlantic salmon varies greatly based on the location, population and success in the sea phase of its life but is between 1-25 kg at 45-130 cm long [12]. When it approaches coastal water and swims towards the fjord and river, it is swimming at a depth of 1-5 meters taking occasional deeper dives before returning to this cruising depth [12]. After reaching the river it swims against the stream trying to reach their mating areas, starting the cycle anew. The Atlantic salmon is iteroparous, meaning it can spawn several times in its life, compared to the most Pacific salmon species who are semelparous and dies after spawning [12].

Farmed Atlantic salmon has a shorter life cycle compared to their wild counterparts. It is hatched in hatcheries on land where it will go from alevin to fry, parr and smolt within (underyearling), or after (yearling), one year depending on the strategy of the facility and customer [20]. The smolt is transferred out to sea cages where it will spend between 16-24 months reaching a weight between 2-8 kg before being slaughtered [21]. However, during its time in sea it is affected by many mortality factors as wild Atlantic salmon is, albeit the causing factors in many cases are different.

In the sea phase the mortality of farmed Atlantic salmon is, amongst other factors, caused by diseases like cardiomyopathy syndrome, heart- and skeletal muscle inflammation along with pancreas disease and gill infections [2, 8, 19, 22]. Diseases in sea cages is a severe event if it occurs, and can have long lasting effects on the salmon population [2]. The sea cages are known to act as reservoirs for both diseases like pancreas disease [19], but also for the parasitic salmon louse *Lepeophtheirus salmonis* [23]. Farmed salmon is also vulnerable to toxic algae blooms and other environmental factors like decreased oxygen content in the water [2]. This is due to not being able to escape the sea cages, unlike wild fish who can simply dive or swim away from an area with unfavourable environment.

Most fish farms for Atlantic salmon in Norway are in or near fjords, placing them in proximity of the rivers inhabited by wild Atlantic salmon populations [24]. Their potential role as a reservoir for contagious diseases and parasites are often discussed and tied to the decline in wild salmon in areas associated with fish farms [23, 25]. They can act as reservoirs due to the high stocking density and they are protected from predators who could have ended the infection cycle. The stocking density in fish farms, up to 200 000 individuals in each sea cage [26], means less than three sea cages would hold the entire Norwegian population of wild salmon in them [25]. In other words, there is an abundance of hosts for diseases and parasites concentrated in one place.

1.3 The salmon louse

There are two parasites from the family Caligidae who has Atlantic salmon as their host, *Lepeophtheirus salmonis* and *Caligus elongatus*. Caligidae belong to the copepods, a group of small crustaceans, and are commonly called sea lice [27]. They are both parasitic species, and the Atlantic salmon is one of their host species. However, the current regulations only apply for the salmon louse, *L. salmonis*, and the focus will therefore be on *L. salmonis* [28].

L. salmonis has a multi-staged life cycle comprised of 8 stages [27], earlier thought to be 10 [29], each separated by molts. Molting is a process where the exoskeleton of crustaceans is shed and a new one is grown in its stead, allowing for an increase in size of the organism and the time between each molting is affected by temperature [30]. During the 8 stages of growth separated by molts, the life cycle of the salmon louse is spread over 5 phases of movement [31-33]. These are: two planktonic stages of nauplius larvae after hatching, one infective stage, two attached chalimus stages, two mobile pre-adult/adult stages and one adult stage [34]. The cycle starts as eggs in the egg sacs of a mature female salmon louse attached to an Atlantic salmon, or other salmonid species, where the eggs will grow over a period of time [33]. Johnson and Albricht 1991 found this period to be temperature dependent varying from 17,5 days at 5 °C down to 5,5 days at 15 °C [31], but another study found it to be 30-40 days at 9 °C and 10-12 days at 11,5 °C [35]. However, both studies show that temperature shortens the development times significantly.

During their lifespan, each mature female can produce up to 6 pairs of egg sacs (12 egg sacs in total), each containing between 100-965 eggs dependent on egg sac length [36], time of year [37] and wild or farmed Atlantic salmon [36]. A quick calculation finds the total number of eggs from 1 mature adult female *L. salmonis* to be between 1200-11500 eggs. The current regulation regarding allowed number of mature female *L. salmonis* says no more than 0,5/0,2 louse per Atlantic salmon depending on the time of year [28]. In a sea cage of 200 000 salmon, the maximum allowed number of mature adults equates to between 40 000 and 100 000 mature lice. Throughout their lifespan of up to seven months, in laboratory conditions [38], this could translate to a potential egg production between 48 million to 1,15 billion eggs in total. The survival rate from nauplii to mature adult is unknown and based on estimations, but the high number of eggs itself supports the regulation in number of allowed mature adults per fish in each sea cage.

The salmon louse adult males reach a size of around 6 mm, while the females reach a length of around 11 mm, without their egg sacs trailing behind them [39]. The effect of an infection of *L. salmonis* on Atlantic salmon depends on what stages of the lice that is infecting. It is mainly when the lice feeds that cause harm to the host, the movements of the antenna and “lips” of the lice cause superficial damage that erodes the epidermal layer [40]. This causes reactions centred around the local area [41]. Movement of the lice along the body of the host causes little or no apparent damage [42]. The feeding action itself is what causes the most damage, feeding on the epidermis of the skin [33]. Over time this loss of epidermis evolves into open sores, scar tissue and damage to fins and respiratory tissue [33]. Open sores and scar tissue not only lower the commercial value, can also open the fish up for possible secondary infections [42].

In addition to the superficial effects of an infection of salmon louse, it also causes internal reactions. As few as 11 chalimus larvae have shown to cause detrimental effect on trout smolt of 15 grams in a experimental conditions [43]. Over 30 mobile pre-adult stage have the potential to cause lethal damage to 40 gram trout smolts [43]. Holst et al. 2003 examined over 3000 salmon smolts and found no individuals with more 10 than pre-adult lice, pointing to similar numbers in Atlantic salmon [44]. The infection causes reactions in the form of increased plasma

cortisol [45], reduced osmoregulatory ability [46], reduced growth [45] and weakens the non-specific part of the immune system [34].

It is during their migration, both the outgoing and returning phase, passing through areas associated with fish farms the wild salmon is most vulnerable to infection of salmon louse [34]. As mentioned earlier the salmon on their migration to and from the sea swim relatively close to the surface at between 1-5 meters. This puts them in the same depth zone as the infective stage of salmon louse seek for hosts to feed on in [47]. Depending on the region of Norway, up to 30 % of wild Atlantic salmon smolt is estimated to be killed by an infection of salmon louse [2]. Farmed Atlantic salmon acts as a reservoir for salmon louse, it is therefore crucial to have control over the number of salmon louse in sea cages of Norwegian fish farms to achieve the sustainability needed for future growth [7, 8].

1.3 Controlling the population of *L. salmonis*

According to regulation 5. December 2012 nr. 1140 about combating the salmon louse in aquaculture facilities the highest average number of mature female lice allowed per salmon in each sea cage is 0,5. During parts of the spring this decreases to 0,2 mature female lice [28]. To reduce the amount of salmon lice the farmed salmon is put through a process called delousing. There are several ways to go about this, using methods from different categories including: chemotherapeutants, freshwater baths, mechanical treatments and thermal treatments [2]. Mechanical treatments use different principles like brushes, water jets or water currents. The thermal treatments include the Optilicer and Thermolicer.

Up until 2014 the delousing process was done through baths or diets containing chemotherapeutants [48]. In this procedure the bottom weight of the sea cage is removed, and a delousing tarpaulin is put around and underneath the whole sea cage. Ropes with tubes entwined in them are stretched from one side of the sea cage to the other. These tubes will supply and disperse the chemotherapeutant and extra oxygen throughout the treatment period. The tarpaulin's purpose is to contain the chemotherapeutant inside the sea cage for the duration of the treatment. When the treatment is complete, the tarpaulin is removed. Another way of

doing it is through crowding and pumping the fish aboard a wellboat for treatment in its wells. The use of chemicals to delouse salmon in sea cages have been heavily disputed for its effect on the salmon itself [49] environment [50] and affecting other crustacean species [51].

Another important point to mention regarding the use of chemotherapeutants is the development of resistance within the population of salmon lice. In their strategy for sustainably aquaculture from 2009 the Ministry of Fisheries and Coastal affairs stated that suboptimal conditions during a chemotherapeutic delousing is a major factor for development of resistance [52]. During a delousing there is no guarantee that 100 % of the lice is killed, nor is this the main goal of a delousing according to NFSA and regulation 5. December 2012 nr. 1140 about combating the salmon louse in aquaculture facilities [28, 53]. The chemotherapeutants affect the lice harder than the salmon, but their toxicity is temperature dependent [54-56]. However, by using a single chemotherapeutant in each treatment and not killing 100 % of the sea louse infestation, there is a risk of developing resistance [57, 58]. This happens over time, as the survivors of each treatment are free to further their genes and the population of resistant louse grows [57].

In 2014 the resistance had spread to most salmon louse populations in the country and across multiple chemotherapeutants [48]. This caused a swift development of novel methods of delousing Atlantic salmon on a commercial scale; freshwater, mechanical and thermal treatments [2, 19].

The freshwater delousing method involves a wellboat with wells of freshwater the fish is pumped into. Atlantic salmon tolerates freshwater well compared to the louse who has shown preference for a salinity over 27 ‰ [59, 60]. Freshwater is an effective delousing method with low pathological effect on the fish [61], but there is a study showing that salmon louse could develop resistance towards the freshwater treatment [62].

Mechanical delousing is a term used to describe three different machines using water jets (FLS system), water jets and brushes (SkaMik) and water turbulence (Hydrolicer) to remove the lice

after pumping the fish onboard specialised boats. According to regulation 17. June 2008 nr. 822 regarding the operation of aquaculture facilities § 20 all methods, installations and equipment can only be used in an aquaculture facility when the consequences for fish welfare has been documented [26]. The FLS system has a documentation of effect and welfare from the Veterinary Institute [63], showing significant difference in scale loss and gill bleeding before/after treatment. SINTEF released a documentation on the Hydrolicer showing significant difference in scale loss and skin bleeding before/after treatment [64]. To the best of our knowledge there is no official documentation for the SkaMik delousing method at the time of writing, only an unpublished report [65].

Thermal delousing of Atlantic salmon is done through either an Optilicer [19] or a Thermolicer [66]. They work on the same principle; the fish is pumped through a wellboat or specialised boat and through a bath of water holding between 28-34 °C water. The documentation of effect and welfare for the Optilicer delousing treatment showed physical injuries from collisions, blunt trauma, rifts in the skin caused by metal and other mechanical injuries [67]. In the documentation of effect and welfare for the Thermolicer there was a significant difference in scale loss before/after treatment along with acute gill bleeding [68].

1.4 The documentation of welfare

There is a focus on welfare in the documentation of delousing procedures. How is welfare defined, and how is it applied, in an aquacultural setting? Welfare is a term that is hard to describe and define, and varies depending on the perspective and background of the viewer [69]. There are three ways that is most commonly used to define whether an animal is in a good state of being; the function based, nature based and feelings based definition of welfare [69].

The first way to define welfare, the function based one, is based on the body of the animal [70]. This definition assumes that an animal that is feeding and growing well is experiencing good welfare, but it also assumes the opposite; an animal that is immunosuppressed or has a reduced life expectancy is experiencing poor welfare [70]. This definition focuses solely on the functions of the bodily systems, ignoring the potential for poor welfare in for instance, social

animals kept alone [69, 71, 72]. Furthermore, proving good welfare through bodily functions like plasma cortisol and heart rate is difficult to interpret, since their values are altered by both positive and negative interactions and influences [72]. Breeding programs can give genetics that shows good growth while ignoring the mental state of the animal [72].

The second way to define welfare, the nature based one, is based on the idea that an animal living like they do in nature, with freedom to express themselves and their full range of natural behaviour is in a good state of being [72-74]. This ignores any and every form of suffering. To put it bluntly, if the salmon is free to swim, it does not matter if an otter, bear or a predatory bird have taken a chunk out of it, it is in a good state of being. A point to mention regarding the sea phase of Atlantic salmon, does it swim continuously because it is tracking food and stop when it finds food? Is it natural for it to swim continuously, or is it fine to stand still if it has food? Does this mean the confinement in sea cage is good welfare, or bad [69, 75, 76]?

The third way to define welfare, the feelings based one, is based on the principle that an animal in good physical shape can experience bad welfare through feelings of pain and fear [69, 77]. This assumes that higher feelings other than instincts like hunger and fight or flight reactions can be found in animals, or more specifically fish in an aquacultural setting. It also assumes that an animal can be visibly injured and still experience good welfare as long as that injury does not cause a feeling of pain [69]. Does this definition of welfare still apply to animals that cannot feel pain? Can fish feel pain? Does an absence of pathology equal good welfare, if it does not elicit a feeling of pain, if we follow this definition? To avoid a moral debate, we must assume that animals can in fact feel pain and that we should treat them as such.

The law of 19. June 2009 nr. 97, Animal Welfare Law, § 3 states that all animals embraced by the statute has an intrinsic value independent to their eventual economical value for humans [78]. It states further that the animal keeper, in this case the fish farm, are obligated to make sure all methods, equipment and technical solutions applied are suited to maintain the welfare of the animal [78]. So, how does one measure animal welfare in aquaculture? The three

definitions mentioned above can conflict with each other, creating the need for a different or specified definition on welfare.

Stien et al. 2013 defines welfare as “the quality of life as perceived by the animal themselves” [79]. This relies on the assumption that fish can experience different states of welfare, and is based on results from studies on “nervous systems linked with emotion, memory, spatial relationships, primary consciousness, reward, cost-benefit estimation and decision making” [79]. The Salmon Welfare Index Model is comprised of a selection of welfare indicators that, when combined, can make an overall welfare assessment of Atlantic salmon in fish farms [79]. Welfare indicators can provide information about how fulfilled an animals needs are, they can be animal or environmental based and the ones chosen were selected for their gradeability [79, 80]. In the model, scientific knowledge of animal physiology and behaviours is applied to surmise an individual’s welfare state [79, 81]. In “Welfare indicators for farmed Atlantic salmon: tools for assessing fish welfare” the welfare indicators are collected into a book format for ease of use in the aquaculture industry [80]. Furthermore, welfare indicators can be divided into operational (indicators realistically used on the farm) and laboratory based welfare indicators (requiring access to a laboratory) [80].

1.5 The histological changes

Skin bleeding and scale loss are two examples of welfare indicators. On the other hand, to assess effects on the fish not seen macroscopically one can use histological examination. Such examinations of tissue samples taken from salmon after a, for instance, Thermolicer treatment will show the microscopical changes. In the gills this is visible as aneurisms caused by burst blood vessels and lifting of the lamellar epithelia [68]. In the skin the epidermis was either lost or thinned out, oedema in the scale pockets and/or a loss of scales. The epidermis is where the mucusin producing cells lie [82]. These cells are responsible for the production of the mucus layer covering the epithelial surfaces of the fish. The cells produce either acidic glycoproteins, neutral glycoproteins or a mix of the two [83].

Staining sections with Alcian blue periodic acid Schiff (AB-PAS) instead of the hematoxylin & eosin (HE) stain used for normal histopathology, will colour the mucus cells blue (acidic) or red/magenta (neutral) [83]. Mixed cells are coloured lilac. Since the cells are located at the top of the epidermis, they are highly exposed to damage and injuries. The production of glycoproteins in the mucus layer have been shown to change under the effect of stressors or environmental factors [84]. A consequence of the removal or thinning of epidermis is a weakening of the mucus layer protecting the fish from pathogens.

A paper published by a diagnostic laboratory showed microscopical changes in organs not usually sampled: thymus, nasal cavity and brain tissue [85, 86]. In the thymus this was formation of cavities of the thymus tissue, increase in mucus cells, focal bleeding and hemosiderosis. Sections of nasal cavity showed a metaplasia of the epithelia into mucus cells and focal bleeding. The article acknowledges that some of these are also possible artefacts caused by the processing, but oedema fluid and blood found in the scale pockets support their claim of it being caused by the treatment instead of artefacts.

To summarize, there are several steps in the treatment process that can cause injury and stress to the fish. It is already explained that this causes damage to different organs, but what happens when the fish undergoes treatments without time to heal in between? In the fish farming industry today, there is often a need for delousing treatments scheduled shortly after one another to comply with the regulation for the allowed number of salmon louse per fish [24]. Thermolicer is a relatively new method of delousing salmon, having only been used for the last 4-5 years. In this time there have been, to the best of our knowledge, few studies on the microscopical effects of Thermolicer treatments. What is known is that Atlantic salmon's ability to heal from injuries is dependent on the ambient sea temperature [87].

1.6 The healing process

After being injured and escaping whatever caused the injury, in this case the Thermolicer, the regeneration begins. It starts by a migration of different cell types including neutrophils, macrophages, endothelial cells and keratinocytes. These cells promote the regrowth of the

epithelium layer and blood vessels while also combating the possibly invading microorganisms [88]. An important part of this regrowth is scar tissue. It is formed by fibroblasts producing collagen which is a key component in the extracellular matrix, a process which is temperature dependent [87, 88]. The metabolic rate of an animal changes with its temperature, and since the Atlantic salmon is ectotherm this means its body temperature conforms to the ambient temperature around it [89, 90].

The effects of a lower sea temperature will therefore mean it takes longer for an Atlantic salmon in a fish farm in colder water to heal compared to one living in warmer water. This can affect how close together two delousing operations can be scheduled. Another factor to consider is stress' effect on healing. According to Sveen et al. 2018 the low temperature delays the repair of the epidermis, while the stress hormone cortisol delays repair of the dermis [91]. A stressful situation has also been proven to cause decreased immune function and changes in the gene expression of Atlantic salmon [92].

1.7 The gene expressional effects

As stated earlier, the processes the fish is subjected to as part of a Thermolicer treatment is stressful. During the exposure to a stressor the body reacts in several ways, one of which is changes to the gene expression of a variety of genes [93]. The chosen genes to examine the effect of a Thermolicer treatment on are interleukin 1β, interleukin 6, tumor necrosis factor α, heat shock protein 70 and complement factor C3. A quantitative real time polymerase chain reaction (RT qPCR or just qPCR) analysis of head kidney- and gill samples can be used to measure the relative expression of the genes by comparing the CT-values [94].

The purpose of a qPCR-analysis is to amplify the amount of DNA through several cycles of denaturation, annealing and extension. During the denaturation stage the double stranded cDNA, made from isolated RNA in tissue samples, is heated up and split into single stranded DNA. The temperature is lowered during the annealing stage to facilitate the primers binding to the DNA. Last stage of the amplification cycle is the extension step: the temperature is

increased and a new strand of DNA is made by a polymerase enzyme using the single stranded DNA as a template [95].

By adding a fluorescent reagent that binds to the DNA strand called SybrGreen, the machine can measure the increase in DNA. This is made possible by the SybrGreen where the increase in fluorescence is proportional to the increase in DNA made by the amplification cycles [94]. The number of amplification cycles need to reach a certain point of fluorescence, and therefore a certain amount of DNA, is displayed as each sample's Ct-value. A lower Ct-value indicates a higher expression of the gene since it took fewer cycles to reach the threshold and vice versa [94]. The Ct-values can then be used to compare the relative expression of genes in the samples through different methods of Ct-analyses.

Using a reference gene allows different groups to be compared by adjusting the threshold of the two qPCR-runs [94]. A reference gene is a gene whose expression is overall constitutively expressed in all genes and tissues [96]. Elongation factor (EF1-α) was chosen because its expression is stable between organs. According to Ingerslev et al. 2006 the expression of EF1-α only varied by a twofold change in expression maximum between gill and head kidney, and is therefore a suited housekeeping gene to use [96].

1.7.1 Interleukin 1β & interleukin 6

Interleukin 1β (IL1β) and interleukin 6 (IL6) are cytokine proteins and part of the immune system. They are both pyrogens, which means they are pro-inflammatory proteins. An area with elevated concentration of IL1β shows an increased recruitment of phagocytes and their phagocytic and lysozyme activity, increased expression of TNFα, and it also activates the expression of immune suppressing genes in Atlantic salmon [97]. It is upregulated after stressful events [92].

IL6 stimulates growth of macrophages and stimulates B-cells to maintain their expression of IgM over time. IL6 can also act anti-inflammatory to maintain the haematopoiesis [97]. In mice

it plays a role in wound healing, but this function is not proven in salmon [98]. It has also been proved to have a role in the increased blood pressure after acute stress in mice, which increases the risk of brain haemorrhaging [99]. As stated by Gismervik et al. in 2019, the collisions might not be the sole cause of brain haemorrhaging. The hot water in the treatment or the stress itself might have an influence as well [86]. The concentration of plasma-IL6 increases rapidly after physical activity in humans [100]. This increase in concentration is likely not caused by expression changes since changes in expression is not near instant like Nielsen et al. 1996 saw.

1.7.2 Tumor necrosis factor α

Tumor necrosis factor α (TNFα) has several overlapping functions with IL1β and is one of the first immune genes expressed at the start of an infection in Atlantic salmon [97]. Its function is to activate macrophages and increases their killing ability while promoting their survival and restricting bacterial growth inside them [97]. In trout it plays a role in both T-cell mediated immunity and the innate immune system [97].

1.7.3 Heat shock protein 70

Heat shock proteins are proteins found in all organisms, including fish. One of their main functions is to maintain the homeostasis and assisting in correct folding of proteins inside the organism [101]. Their function in the homeostasis and protein folding means they are already highly expressed during normal cell growth. Proteins, and their folding process, are generally vulnerable to heat due to misfolding and denaturation risk. One function of heat shock protein 70 (HSP70) is to refold misfolded proteins and its expression is upregulated when temperature increases [102]. This points to heat shock proteins being an important factor in an organism's thermal tolerance.

Another factor that points to HSP's connection to thermal tolerance is the fact that HSPs inhibit caspase activation and apoptosis of its host cells [103]. This function helps the cell survive after damaging stimuli and stress. There are several factors that regulates their expression including temperature changes and inflammatory stress [101]. In a study on acclimation temperature and heat shock in *Gillichthys mirabilis* there was a significant upregulation of HSP70 regardless of

acclimation temperature [104]. To summarize, the HSP70 is constitutively expressed due to its important functions and is upregulated during stressful encounters.

1.7.4 Complement factor C3

Complement factor C3 (C3) is a protein in the complement system of the immune system. Its main function is its role in activation of the classical and alternative complement system [105]. This is used in the defence of its host against pathogens, but also clearing cellular debris and suppressing tumor cell growth [105]. Its presence in salmon is well established [106]. In humans it is regulated by the concentration of IL1β, IL6 and TNFα [107]. Seeing as IL1β, IL6 and TNFα are all upregulated by stress, this means that C3 is also upregulated after an encounter with a stressor.

The genes were all selected due to their role in the salmon's reaction to a stressor. How does one separate the warm water's effect on the salmon from the rest of the stressors like crowding, netting, pumping etc.? In this case the stressor we want to know the effect of is the 30 second treatment with 34 °C water. The histological examination and the qPCR-analysis of the genes is an attempt to identify this effect. One way to do this is to complete the treatment in a controlled setting with two experiment groups; one treated in warm water and one treated with cold water. Comparing the two groups will let you see what differed between them. It is plausible the difference is the effect of the warm water. A 34 °C warm-water treatment will not include any of the unknown variables that influence the results of a full-scale Thermolicer treatment. To include these unknown variables a field treatment with a Thermolicer is included for comparison.

1.8 Master's thesis' goal and objectives

Goal:

- To examine the histological, mucosal and gene expressional effects of a 30 second 34 °C warm-water treatment on Atlantic salmon immediately after and three weeks post-treatment compared to a full-scale Thermolicer treatment in the field.

Objectives:

- Compare the mean welfare indicator scores from a 30 second 34 °C treatment compared to a full-scale Thermolicer treatment in the field to look for differences in the macroscopical effect on the fish.

- Assess the effect of a 30 second 34 °C treatment and full-scale Thermolicer treatment on important surface organs using histology.

- Examine potential differences in mucus cell acidity before and after being exposed to warm water in sections of skin and gills.

- Examine differences in gene expression of Atlantic salmon going through a 30 second 34 °C and full-scale Thermolicer treatments.

2. Methods

2.1 Experiment groups and data

The experiment was designed to consist of two parts; a controlled experiment and a field experiment. The controlled experiment was completed at the Institute of Marine Research's facility at Matre outside of Bergen in May 2019. This consisted of Atlantic salmon acclimated to 8 °C before being split into two groups. One small group would undergo a 30 second 34 °C warm-water treatment. The second group would go through the same process, but at 8 °C with the purpose of removing high water temperature as a cause of injury. Three weeks later the warm water treated fish was euthanized with Tricaine (Pharmaq) along with a part of the cold-water group to act as control.

To examine the effects of a 30 second 34 °C warm-water treatment, the fish was transferred from its holding pen (8 °C) and into a perforated box before being submerged in the 34 °C water. A temperature difference of 26 °C, or $\Delta t = 26$ °C. The same process was done for the cold-water treatment, but in 8 °C water.

All fish taken from the controlled experiment and examined in this thesis underwent at least one cold-water treatment. To check for long-term injuries after the warm water treatment one group underwent a warm water treatment and rested for three weeks before sampling. This group is named warm-water long-term group, or WWLT for short. For comparison purposes, a group underwent another cold-water treatment at the same time as WWLT went through warm-water and was sampled after three weeks rest. This is the cold-water long-term group and it is checking for the eventual effect of the handling the fish undergoes during the thermic delousing process. After three weeks the long-term groups were netted over to a bath with an overdose of Tricaine (Pharmaq) for euthanization.

The last two groups in the controlled experiment are the cold-water- and warm-water short-term groups, named CWST and WWST for short. In total these two groups had undergone three cold-water treatments (CWST) or two cold-water and one warm-water treatment (WWST). The

warm-water group's purpose is to check for the short-term effect of warm-water treatment, whilst the cold-water group is acting as a control.

Atlantic salmon undergoing the field treatment were also divided in two groups; field treated short-term (FTST) and field treated long-term (FTLT). They both underwent a thermic delousing treatment using Thermolicer at 29 °C, a Δt = 24,5 °C from the 5,5 °C sea temperature. The sea farms are both located at Senja in northern Norway, but the farms wished to remain anonymous. Unlike the controlled experiments the field groups are not from the same population. This was due to several factors, all of which will be discussed later.

A summary of all group names, abbreviations, treatment temperature and weight are shown in table 1. Difference in weight between the long-term groups are caused by the three weeks between the treatment times. The temperature difference from acclimation temperature and treatment temperature was 26 °C for the controlled experiment and 24,5 °C for the field experiment. Both groups were vaccinated. The controlled experiment groups were vaccinated with Aquavac 6 vet. The fish farms did not disclose what vaccine they had vaccinated with.

Table 1: Division of the salmon into different experiment groups. Cold and warm water groups were hatched, reared and treated at Institute of Marine Research's facility at Matre. The field treated salmon belonged to a fish farm who wished to be anonymous. Standard deviation and number of fish weighed was not received from the fish farms.

Group	Abbreviation	Treatment temperature	Avg. weight (± SD)
Cold water short-term	CWST	8 °C	1657 ± 450,9
Warm water short-term	WWST	34 °C	1742,8 ± 383,5
Cold water long-term	CWLT	8 °C	1339 ± 254,4
Warm water long-term	WWLT	34 °C	1509,4 ± 276,6
Field treated short-term	FTST	29 °C	3,2
Field treated long-term	FTLT	29 °C	3,6

2.2 Welfare indicators

Before sampling the fish were welfare scored using the welfare indicators in table 2. The scoring system works on a 0-3 scale [80]. A 0 on the scale means there were no signs of lowered welfare detected, while a score of 3 means there were large areas affected/severe changes seen. The welfare scoring in the controlled experiment were scored by Lene Moltumyr og Jonatan Nilsson, researchers at the marine research facility. In the field experiment the fish were scored by a veterinarian from Marin Helse, a fish health consulting firm, using a scoring scale based on the one the Veterinary Institute used under the documentation of Thermolicer as a delousing method [68]. The results from the two different scoring scales are similar enough to be integrated.

Table 2: The two welfare indicators used to macroscopically score the fish before and after treatment, taken from the FISHWELL-project [80]. The scale goes from 0-3 in ascending severity.

Welfare indicator and score	**Skin bleeding**	**Scale loss**
0	None detected	None detected
1	Petechiae in the skin of the gut	Loss of individual scales
2	Larger areas with petechiae	Small areas with no scales
3	Recently bleeding areas, often with significant scale loss and wounds	Large areas with no scales

2.3 Data material

2.3.1 Controlled experiment

Table 3 shows the number of fish and total number of organs sampled from the controlled experiment groups. The difference between short- and long-term groups is due to a wish to have more warm-water treated samples than cold water ones. This gave a higher n to use in statistics and more samples to study for short-term injuries.

Table 3: Overview of the number of fish, and organs, sampled from the controlled experiment groups. The difference in number of individuals in cold water long-term and warm water long-term was due to a wish to have more warm water treated than cold water treated. Eyes, kidney and nostrils were sampled, but not examined apart from testing the protocol due to time constraints/priorities.

Organs/group	CWST	WWST	CWLT	WWLT
Gills	16	16	5	13
Skin	16	16	5	13
Thymus	16	16	5	13
Pseudobranch	16	16	5	13

2.3.2 Field experiment

Table 4 shows the number of fish and organs sampled in total from the field experiment groups. There are two fewer individuals in the FTST group compared to FTLT. This was not due to a shortage of fish or a wish to have more or less of one group, there was simply not more time left before the boat back to shore left and sampling had to stop.

Table 4: Overview of the number of fish, and organs, sampled from the field treated fish. Eyes, kidney and nostrils were sampled, but not examined due to time constraints/priorities.

Organs/group	FTST	FTLT
Gills	8	10
Skin	8	10
Thymus	8	10
Pseudobranch	8	10

2.4 Organ sampling

Organ sampling was performed similarly for all groups in the experiment. All organ samples were stored on 4 % neutral buffered formalin. All the samples were stored at room temperature at the Norwegian College of Fishery Science until they were further processed. For the purpose of qPCR-analysis samples of gill, thymus and head kidney was stored on RNA-later at -20 °C awaiting processing. This was not done for FTLT because it could not act as a control for FTST due to it being a separate population. It could have been analysed on its own, but there were no samples put on RNA-later to save costs on material that was not going to act as a control. As the controlled fish was being sampled, they were also sexed. This was to look for differences between the heat tolerance between the sexes, as was found in guppies during a temperature tolerance test [108].

2.4.1 Nostril

The left nostril of the controlled experiment fish was removed by making a deep incision from the snout to the eye pit along the medial plane. This was repeated on the horizontal plane, making the removed sample have a rounded pyramid-like appearance including the nasal pit.

2.4.2 Eye

Removal of the eye was done by carefully cutting around the eye, inside the eye pit, with a scalpel. Being careful not to puncture the eye the scalpel was pushed in and used as a lever to gain access to the optical nerve behind it. After cutting the nerve and surrounding tissue the eye was removed.

2.4.3 Thymus

Thymus is located at the cross section where the operculum is attached to the top of the gill chamber and was cut out in a pyramid shape using a scalpel.

2.4.4 Gill

Gill samples were cut from the second gill arch on the fish’s left side using a scissor.

2.4.5 Pseudobranch

Pseudobranch was cut along its edges with a scalpel and removed with forceps.

2.4.6 Skin

Skin samples were taken across the lateral line below the dorsal fin of each fish. The piece cut out was 2 cm long, 0,5 cm wide and 1 cm deep.

2.5 Protocols

2.5.1 Tissue processing

To process the samples stored on formalin they were moved into a tissue processor (Citadel 2000, Shandon). Table 5 shows the protocol used for processing the organ samples from both the controlled and field experiment.

Table 5: The protocol used for tissue processing in a Citadel 2000 tissue processor (Shandon).

Protocol for tissue processing	
96 % EtOH	2 hours
96 % EtOH	2 hours
Abs. EtOH	2 hours
Abs. EtOH	2 hours
1:1 100 % EtOH & Histoclear	1 hour
Histoclear	1 hour
Histoclear	1 hour
Histoclear	1 hour
1:1 Histoclear & paraffin wax	1 hour
Paraffin wax	Minimum 1 hour
Total: 14 hours minimum	

A Leica EG 1150H paraffin dispenser station was used to embed the tissue cassettes using Histowax (Histolab) at 60 °C. The cassettes were refrigerated on a cooling element and stored in a refrigerator. After processing and embedding the samples they were sectioned using a Leica RM 2255 microtome at 4 µm and put in a heating cabinet holding 64 °C for one hour to deparaffinize.

2.5.2 Hematoxylin & Eosin staining

After deparaffinization the sections of gill, pseudobranch, thymus and test sections of eye and nostrils were stained with hematoxylin and eosin using the protocol found in table 6. This protocol is the standard protocol used for staining at the Norwegian College of Fishery Science. The staining process was done manually.

Table 6: Protocol used for hematoxylin and eosin staining sections following an hour of deparaffinization at 64 °C.

Protocol for HE-staining	
Histoclear	6 min.
Abs. EtOH	90 s.
96 % EtOH	90 s.
96 % EtOH	90 s.
Rinse in water	90 s.
Hematoxylin	90 s.
Hematoxylin	90 s.
Rinse in water	90 s.
Rinse in water	90 s.
Eosin	90 s.
96 % EtOH	90 s.
96 % EtOH	90 s.
Abs. EtOH	90 s.
Abs. EtOH	90 s.
Histoclear	90 s.
Total: 27 min	

2.5.3 Alcian blue periodic acid Schiff

AB-PAS staining was done to categorize and counting mucus cells in sections of gills and skin. Table 7 shows the staining protocol for AB-PAS staining sections of skin and gill. This protocol is taken from the veterinary institute of Harstad's diagnostical laboratory.

Table 7: Protocol used for AB-PAS staining sections following an hour of deparaffinization at 64 °C. Hydrochloric acid was diluted to 600 µl HCl in 300 ml 96 % EtOH.

Protocol for AB-PAS staining	
Histoclear	5 min.
Histoclear	5 min.
Histoclear	5 min.
Abs. EtOH	5 min.
Abs. EtOH	5 min.
96 % EtOH	5 s.
70 % EtOH	5 s.
Alcian blue	15 min.
Rinse in water	2 min.
0.5 % Periodic acid	10 min.
Rinse in water	30 s.
Schiff's reagent	15 min.
Rinse in water	5 min.
Hematoxylin	30 s.
Rinse in water	1 min.
Hydrochloric acid	2 s.
Rinse in water	30 s.
Bluing (warm water)	1 min.
Rinse in water	20 s.
Total: 1 hour and 16 min.	

All chemicals, dilutions and their suppliers used in the protocols can be found in table 1 in appendix.

2.6 Overview of samples

2.6.1 Scoring system for histology

After processing, sectioning and staining the sections they were histologically examined and scored using a light microscope. The sections were scored on a simple system of 0-3 based on the percentage of tissue affected; 0 (< 5 %), 1 (5-10 %), 2 (10-50 %) and 3 (>50 %) [109].

2.6.2 Controlled experiment

The number of samples from the controlled experiment that were examined are shown in table 8. Compared to table 3 there are some differences in number of samples and results. This was caused by several factors.

A part of this difference was likely caused by using a general program for processing tissue. The effect this has on the tissue depends on the type of tissue [110]. For instance, sections made from thymus samples have tiny cracks all throughout the section of tissue, a sign of over-processing and exacerbated by poor microtome skills [111]. Skin samples on the other hand, which is a fattier tissue, were under-processed [112]. This is shown as the outer rim of tissue sectioning nicely while leaving a hole where the under-processed tissue lies.

Due to the under-processing issue the skin samples from the controlled experiment were sectioned at the Veterinary Institute (VI) in Harstad. Their recommendation was to submerge the fixated skin samples in Decalc decalcifying fluid (Histolab) for one hour before processing. This decalcifies the tissue making the skin blocks easier to section, but it does not fix the issue of under-processing. Having learnt from this, the skin samples from the field experiment were sectioned at the Norwegian College of Fishery Science.

Another reason was finding the relevant tissue one wanted to examine. Finding the thymus tissue in the samples taken proved to be challenging, only hitting the correct spot in 10 % of the first sectioning and staining session. After repeated sectioning attempts, thymus was still not found in some of the samples. A possible reason is that the area containing thymus tissue was

missed when the sample was taken. Nostrils and eyes were difficult to section, but were tested since pathological changes in the nostril and eye was discovered in the pilot experiment [86].

Table 8: The number of histologically examined and scored samples. Table 5 also shows the test samples from nostrils and eyes.

Organs/group	CWST	WWST	CWLT	WWLT
Gills	15	15	4	10
Skin	15	11	3	10
Thymus	13	13	2	10
Pseudobranch	13	13	3	8
Nostrils	0	0	4	4
Eyes	0	2	0	1

2.6.3 Field experiment

Skin samples from the field experiment were submerged in Decalc decalcifying fluid (Histolab) for one hour before processing in the Citadel 2000 tissue processor (Shandon). There were only 2 sections of skin from the FTLT group that had an epidermis section large enough to count and categorize. It is unknown whether this is caused by outside factors or the processing protocols.

Table 9: The number of histologically examined and scored samples.

Organs/group	FTST	FTLT
Gills	8	10
Skin	8	2
Thymus	8	8
Pseudobranch	8	10

2.7 qPCR

Samples of thymus, gills and head kidney was taken from fish in the controlled experiment and from day 0 of field treated fish. The samples were stored at RNA-later in a -20° Celsius freezer at the Norwegian College of Fishery Science. To extract RNA from the tissue the RNeasy 250 mini-kit (Qiagen) and its protocol was used. The protocol from the RNeasy mini kit requires the tissue (30 mg) to be homogenised, this was done in a TissueLyzer II (Qiagen) using 2 ml tubes and autoclaved lead beads. After extracting the RNA its concentration and purity was measured using Nanodrop (Thermo Scientific). The isolated RNA was stored at -80°C.

Using Quantitect Reverse Transcription-kit (Qiagen) cDNA was synthesized from the isolated RNA. In the first step, removal of genomic DNA, the isolated RNA was diluted to 100 ng/µl and 5 µl of the diluted RNA was added to the reaction. The sample strips were incubated at 42 °C for 2 minutes, to activate the gDNA wipeout, before being put on ice. For the rest of the cDNA synthesis the kit protocol was followed for a total volume of 20 µl cDNA. Negative control was made without reverse transcriptase in the reaction. To finish the cDNA synthesis the sample strips were incubated at 42 °C for 15 minutes, 95 °C for 3 minutes and then a 4 °C hold to immediately cool the samples down from 95 °C. The synthesised cDNA was stored at -20°C.

For the qPCR-run, six genes were selected. Elongation factor was used as the reference gene. Interleukin 1β, interleukin 6, tumour necrosis factor α, complement factor 3 and heat shock protein 70 were the five genes run for comparison between the fish groups.

The qPCR was set up using a 96-well tray filled with a Sybr Green-mix containing 10 µl Fast Sybr green master mix (Applied Biosystems), 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM) and 3 µl H_2O. 5 µl cDNA was then added to each well before the tray was sealed with a film. Samples and negative reverse transcriptase controls were run in duplicates. On each tray there were two H_2O-wells to check for the formation of primer dimers and a positive control was run in duplicate to adjust the threshold for comparison between the two plates running the same gene. After the qPCR-analysis was done and the Ct-values were obtained, the delta delta

Ct-method, also known as the $2^{\Delta\Delta Ct}$-method, was used to calculate the relative gene expression differences [95].

2.8 Statistical analyses

In this experiment the significance value is set to 5 % ($p < 0,05$). To calculate the significance of welfare scores and histological scores Welch's t-test was used. Welch's t-test was chosen over Student t-test for three reasons. It is simple and gives good control over type 1 errors and is preferred over Student's t-test in instances where the variance and size of groups are not equal [113]. The calculations were performed in Excel 365 (Microsoft) and graphs were made in GraphPad Prism 8 (GraphPad Software).

3. Results

3.1 Welfare

After the 30 second treatment the fish was scored either immediately or at day 21 post-treatment. Table 10 shows the welfare scores from the cold- (8 °C) and warm-water (34 °C) short-term treated (scored immediately after) and long-term (scored three weeks post-treatment).

Table 10: Mean welfare indicator scores of skin bleeding and scale loss from the cold and warm water treated groups shown with their standard error mean.

Group	Skin bleeding (± SEM)	Scale loss (± SEM)
CWST (n=16)	0.5 ± 0.13	1,06 ± 0.11
WWST (n=16)	0.44 ± 0.13	1,33 ± 0.12
CWLT (n=5)	0.2 ± 0.18	1,0 ± 0.00
WWLT (n=13)	0.54 ± 0.14	1,31 ± 0.13

The results in table 10 shows small, insignificant differences between the groups' skin bleeding scores ($p > 0.05$). CWLT had the lowest skin bleeding score, 0.2. The scale loss scores show that both cold-water groups had a lower scale loss compared to the warm water group, but these differences were insignificant ($p > 0.05$). The difference between the warm-water short- and long-term group is insignificant too, despite three weeks rest post-treatment for the long-term group ($p > 0.05$).

Before and after the Thermolicer treatment the fish in the field treated short term group was welfare scored. Table 11 shows the welfare scores from the field treated short term group that underwent a Thermolicer treatment at 29 °C.

Table 11: Mean welfare indicator scores from the field treated short-term group shown with their standard error mean.

Checkpoint	Skin bleeding	Scale loss
Before Thermolicer	0.1 ± 0.11	0.1 ± 0.11
After Thermolicer	0.2 ± 0.14	0.5 ± 0.18

The results in table 11 shows a small, insignificant increase in skin bleeding score after the Thermolicer treatment compared to before. The difference in scale loss before compared to after the Thermolicer treatment was significant and shows an increase in scale loss mean from 0.1 to 0.5 ($p > 0.05$). A possible flaw in the method of catching fish after the Thermolicer treatment was found and is discussed later.

3.2 Histological scores

The HE-stained sections of gills, pseudobranch and thymus from cold- and warm-water treated short term groups were scored histologically. The scale goes from 0 to 3, but there were no histopathological changes that affected more than 10 % of the tissue sections. Therefore table 12 only shows columns for score 0 and 1, mean score for each organ and the n of each group.

Table 12: Histological scores from HE-stained sections of gill, pseudobranch and thymus of the cold and warm water short-term injuries groups. n differed between the groups and is shown in the table. Scores: 0 (< 5 %), 1 (5-10 %) of tissue affected.

Group	**CWST**				**WWST**			
Organ/score	**0**	**1**	**Mean**	**n**	**0**	**1**	**Mean**	**n**
Gills	14	1	0.07	15	9	6	0.4	15
Pseudobranch	13	0	0.00	13	11	2	0.15	13
Thymus	8	4	0.33	12	8	5	0.38	13

The results in table 12 shows a small, but significant difference in the number of gills scored 1 (> 5 % of tissue affected) between the cold- and warm-water treated short term group ($p < 0.05$). This difference was seen in the amount of hyperplasia and some bleedings. In the pseudobranchs there was a small, insignificant increase in the number of pseudobranchs scored 1 between the cold- and warm-water treated short term groups. There was one bleeding found, and one tear in the tissue that is likely and artefact of the processing. In the thymus there was a small, insignificant increase between the cold- and warm-water treated short term group ($p > 0.05$). This was an increase in mucus cells, one bleeding, one hemosiderosis and formation of cavities.

The HE-stained sections of gills, pseudobranch and thymus from cold- and warm-water treated short term groups were scored histologically. The scale goes from 0 to 3, but there were no histopathological changes that affected more than 10 % of the tissue sections. Therefore table 13 only shows columns for score 0 and 1, mean score for each organ and the n of each group.

Table 13: Histological scores from HE-stained sections of gill, pseudobranch and thymus of the cold and warm water long-term injuries groups. n differed between the groups and is shown in the table. Scores: 0 (< 5 %), 1 (5-10 %) of tissue affected.

Group	**CWLT**				**WWLT**			
Organ/score	**0**	**1**	**Mean**	**n**	**0**	**1**	**Mean**	**n**
Gills	4	0	0.0	4	8	2	0.2	10
Pseudobranch	4	0	0.0	4	8	0	0.0	8
Thymus	2	0	0.0	2	8	2	0.2	10

The results in table 13 shows a small, but insignificant difference in the number of gills scored 1 (> 5 % of tissue affected) between the cold- and warm-water treated long-term group ($p > 0.05$). This difference was a bleed and lifting of the epithelium in one of the two sections and hyperplasia in the other gill section. In the pseudobranchs there was no difference between the two groups. In the thymus there was a small, insignificant increase between the cold- and warm-water treated long-term group ($p > 0.05$). There were only two sections from CWLT where the thymus tissue was located, making the calculation of significance inaccurate. There were no significant differences between WWLT compared to WWST in any organ, but both the gills and thymus was on average scored higher in the group sampled immediately compared to after three weeks.

The HE-stained sections of gills, pseudobranch and thymus from the short- and long-term field treated groups were scored histologically. The scale goes from 0 to 3, but there were no histopathological changes that affected more than 10 % of the tissue sections. Therefore table 14 only shows columns for score 0 and 1, mean score for each organ and the n of each group.

Table 14: Histological scores from HE-stained sections of gill, pseudobranch and thymus of the field treatment short-term injuries group. n differed between the groups and is shown in the table. 0 = none detected, 1 = < 10 % of tissue affected.

Group	**FTST**				**FTLT**			
Organ/score	**0**	**1**	**Mean**	**n**	**0**	**1**	**Mean**	**n**
Gills	7	1	0.125	8	8	2	0.20	10
Pseudobranch	8	0	0.00	8	10	0	0.00	10
Thymus	3	5	0.60	8	5	3	0.38	8

The results in table 14 shows differences between the gill scores from the field treated short- and long-term groups. The mean gill score was higher in the long-term group than in the short-term, despite having 10 days rest after their Thermolicer treatment. The pseudobranch had no scores higher than 0 in either of the groups. The mean thymus score was higher in the short-term group sampled immediately after their Thermolicer treatment. Significance was not calculated as the groups are from two different populations of farmed Atlantic salmon.

3.3 Mucus cells

The results are presented in tukey box plots, where the bottom edge of the box is the 25th percentile (first quartile) and the upper edge is the 75th percentile (third quartile). The whiskers are 1.5 times the width of the [25th, 75th] interval. Outliers are marked as dots outside of the whiskers.

The mucus cells were counted and categorized into either acidic or neutral mucus cells. Figure 1 shows a comparison between the count of acidic and neutral mucus cells in skin sections from the cold- (n=15) and warm-water (n=11) treated short-term groups. The results are made from table 1 and 2 in appendix.

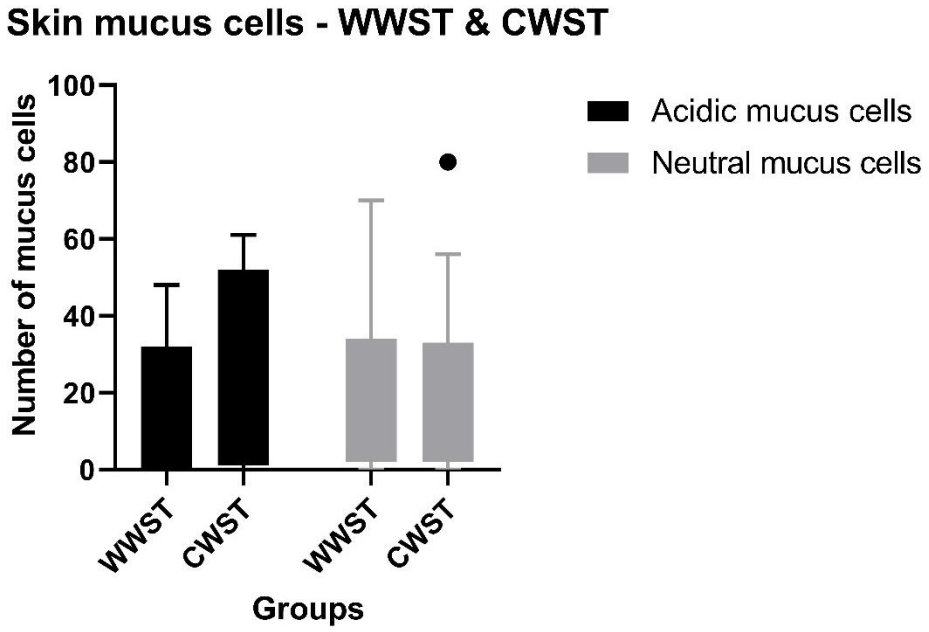

Figure 1: A tukey box plot comparison of the acidity and count of mucus cells in skin sections of warm- (n=11) and cold-water (n=15) treated short term groups. An outlier is marked as a dot.

The results show a mean acidic mucus cell count of 15.3 (SEM ± 5.4) in [0, 34] for WWST and 27.9 (SEM ± 5.8) in [1, 52] for CWST. Mean neutral mucus cell count was 22.4 (SEM ± 6.6) in [0, 34] for WWST and 17.5 (SEM ± 6.4) in [2, 33] for CWST. An outlier at 80 neutral mucus cells in CWST is marked as a dot. There was a seemingly large, but insignificant difference in acidic mucus cells between the warm- and cold-water treated group ($p > 0.05$). There was no significant difference between the neutral mucus cells ($p > 0.05$).

The mucus cells were counted and categorized into either acidic or neutral mucus cells. Figure 2 shows a comparison between the count of acidic and neutral mucus cells in skin sections from the warm-water treated long-term (n=10) and the cold-water treated short-term (n=15) and group. WWLT is compared to CWST due to low n in CWLT (n = 3). The results are shown in tukey box plots made from table 1 and 2 in appendix.

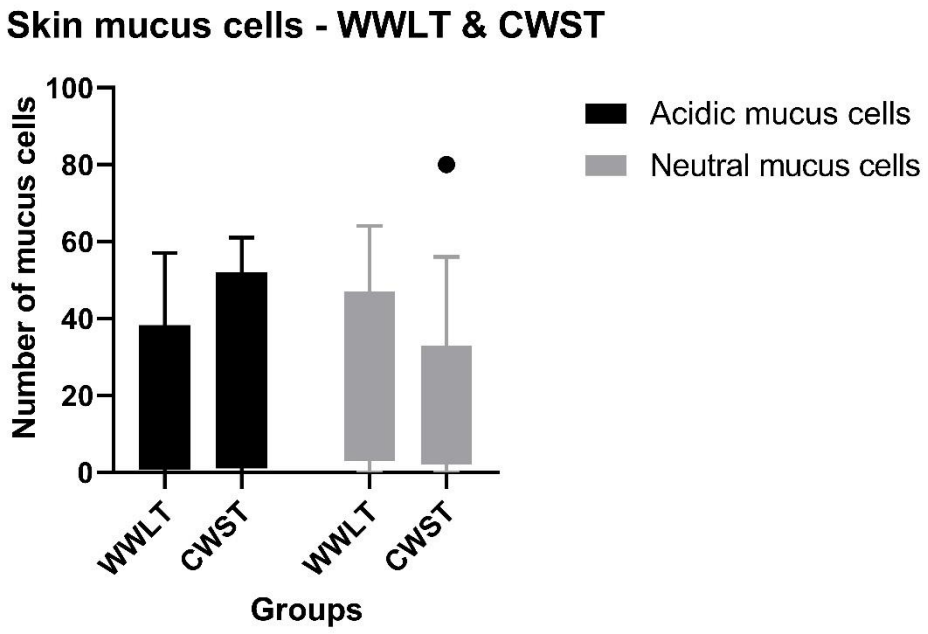

Figure 2: A tukey box plot showing the count and acidity of mucus cells in skin sections from the warm-water long-term group (n=10) compared to the cold-water short-term group (n=15). An outlier is marked as a dot.

The results show a mean acidic mucus cell count of 18.8 (SEM ± 6.6) in [0.75, 38.35] for WWLT and 27.9 (SEM ± 5.8) in [1, 52] for CWST. Mean neutral mucus cell count was 24.5 (SEM ± 7.7) in [0.3, 47] for WWLT and 17.5 (SEM ± 6.4) in [2, 33] for CWST. There was a noticeable, but insignificant difference in the acidic mucus cells between warm-water long-term and cold-water short-term group ($p > 0.05$). An outlier at 80 neutral mucus cells from CWST is marked as a dot. There was a noticeable difference in the neutral mucus cells between the two groups, but this was insignificant ($p > 0.05$). There were no significant differences between the warm-water short- and long-term group ($p > 0.05$).

The mucus cells were counted and categorized into either acidic or neutral mucus cells. Figure 3 shows a comparison between the count of acidic and neutral mucus cells in skin sections from the field treated short-term (n=8) and cold-water treated short-term (n=15) group. FTST was compared to CWST due to low n in the field treated long-term group (n=2). The results are shown in tukey box plots made from table 1 and 2 in appendix.

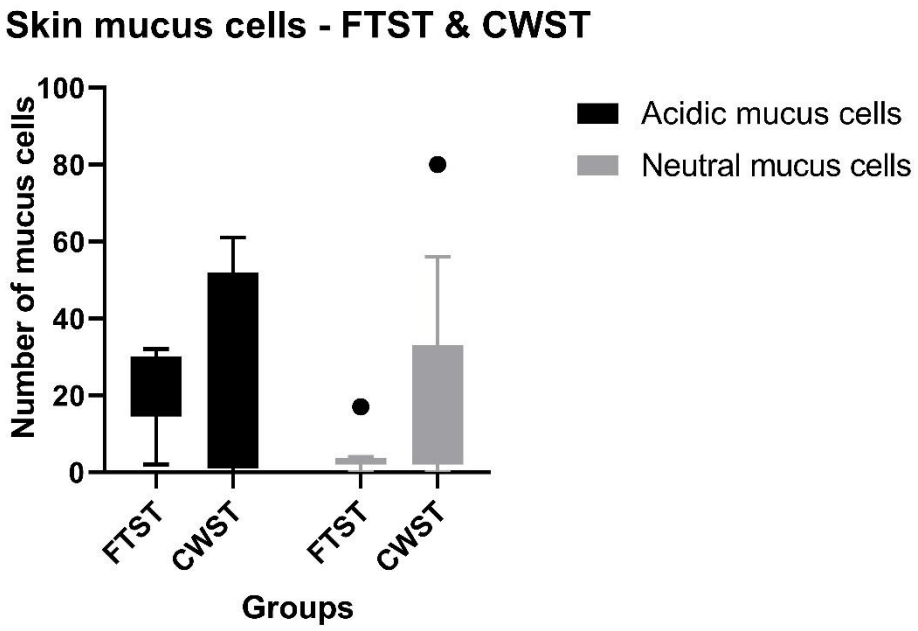

Figure 3: A tukey box plot comparison showing the count and acidity of mucus cells in skin sections from the field treated short-term group (n=8) and cold-water short-term group (n=11). Outliers are marked as a dot.

The results show a mean acidic mucus cell count of 20.9 in [14,5, 30] for FTST and 27.9 (SEM ± 5.8) in [1, 52] for CWST. Mean neutral mucus cell count was 4.3 (SEM ± 1.8) in [2, 3,75] and 17.5 (SEM ± 6.4) in [2, 33] for CWST. An outlier at 17 neutral mucus cells in FTST and 80 neutral mucus cells in CWST are marked as dots. There is a noticeable difference in the acidic mucus cells between the field treated and cold-water treated group, but this was insignificant ($p > 0.05$). There was a significant and large difference between neutral mucus cells in the field treated short term group compared the cold-water short-term group ($p < 0.05$).

The mucus cells were counted and categorized into either acidic or neutral mucus cells. Figure 4 shows a comparison between the count of acidic and neutral mucus cells in gill sections from the cold- (n=11) and warm-water (n=16) treated short-term groups. The results are shown in tukey box plots made from table 1 and 2 in appendix.

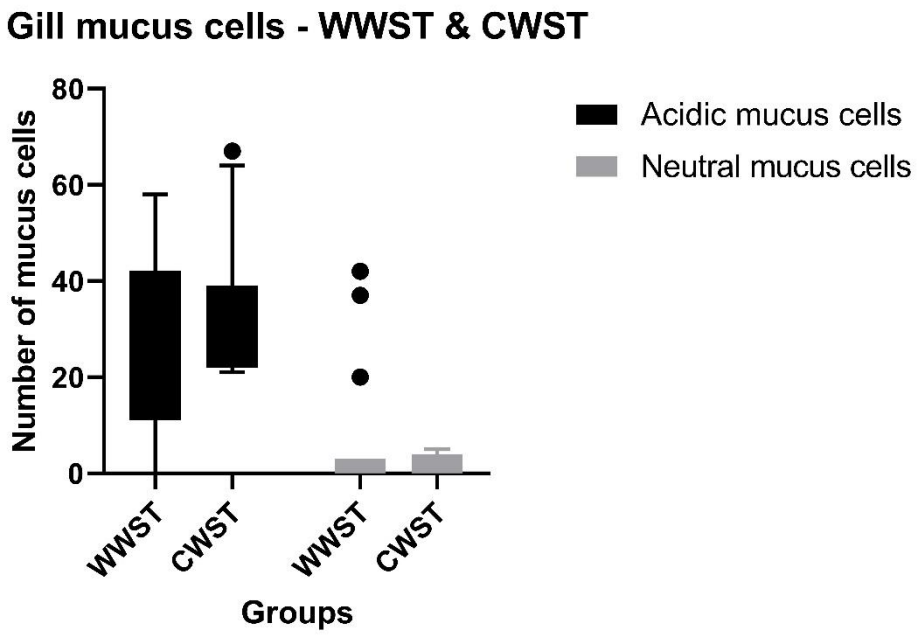

Figure 4: A tukey box plot comparison of the count and acidity of mucus cells in gill sections made from the warm- (n=16) and cold-water (n=11) short term groups. Outliers are marked as dots.

The results show a mean acidic mucus cell count of 27.1 (SEM ± 4.4) in [11, 42.5] for WWST and 34.4 (SEM ± 4.9) in [22, 39] for CWST. Mean neutral mucus cell count was 7.1 (SEM ± 3.4) in [0, 3] for WWST and 2.3 (SEM ± 0.5) in [0, 4] for CWST. An outlier at 67 acidic mucus cells in CWST is marked as a dot. The three outliers in neutral mucus cells of WWST are marked as dots at 20, 37 and 42. There were no significant differences between the warm- and cold-water treated short-term groups in acidic mucus cells ($p > 0.05$). Despite the three outliers in the warm-water treated group there were no significant difference between the two groups in neutral mucus cells ($p > 0.05$).

The mucus cells were counted and categorized into either acidic or neutral mucus cells. Figure 4 shows a comparison between the count of acidic and neutral mucus cells in gill sections from the cold- (n=5) and warm-water (n=9) treated long-term groups. The results are shown in tukey box plots made from table 1 and 2 in appendix.

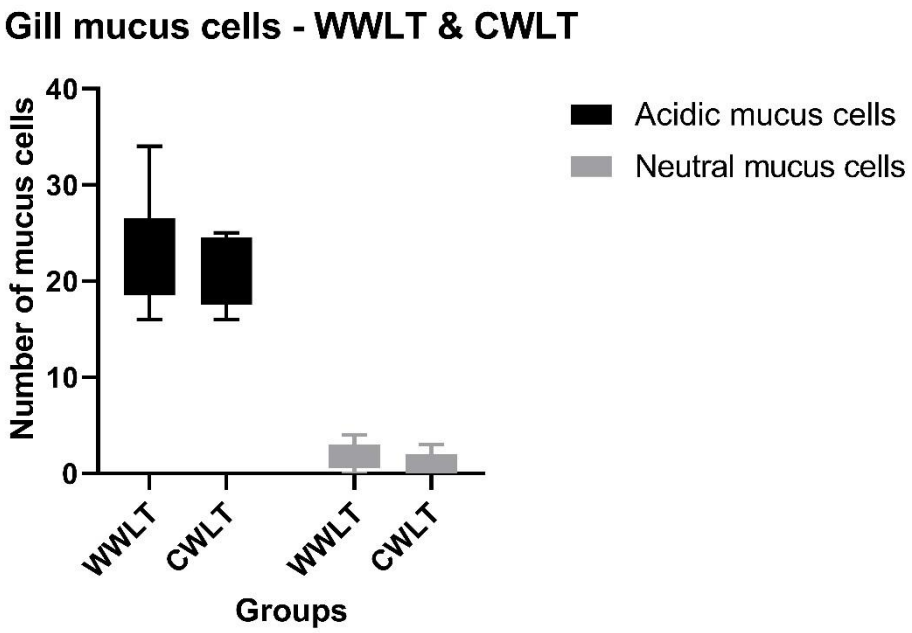

Figure 5: A tukey box plot comparison of the count and acidity of mucus cells in gill sections made from warm- (n=9) and cold-water treated long term groups (n=5).

The results show a mean acidic mucus cell count of 22.9 (SEM ± 1.9) in [11, 42.5] for WWLT and 21.0 (SEM ± 1.6) in [22, 39] for CWLT. Mean neutral mucus cell count was 2.0 (SEM ± 0,5) in [0, 3] for WWLT and 0.8 (SEM ± 0.5) in [0, 4] for CWLT. There was a small but insignificant difference between the warm- and cold- water treated groups three weeks post-treatment in acidic mucus cell count ($p > 0.05$). The difference in neutral mucus cells was insignificant in neither the neutral nor acidic mucus cells ($p > 0.05$). There were no significant differences between the warm-water short- and long-term group ($p > 0.05$).

The mucus cells were counted and categorized into either acidic or neutral mucus cells. Figure 3 shows a comparison between the count of acidic and neutral mucus cells in gill sections from the field treated short-term (n=8) and cold-water treated short-term (n=11) group. FTST was compared to CWST due to low n in the field treated long-term group (n=2) and interest to see short term samples from field compared to controlled experiment. The results are shown in tukey box plots made from table 1 and 2 in appendix.

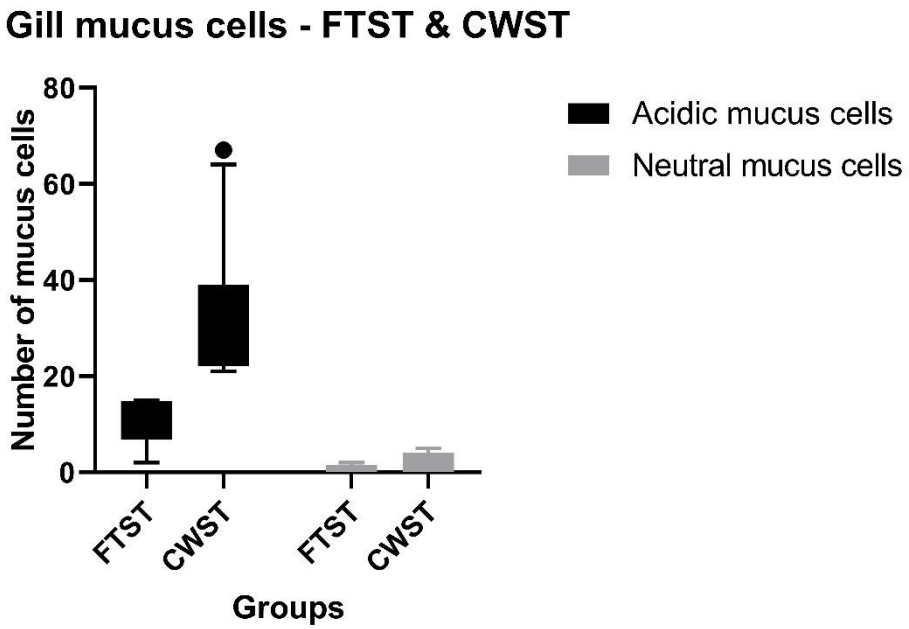

Figure 6: A tukey box plot comparison of the count and acidity of mucus cells in gill sections made from field treated short term group (n=8) and cold-water short-term group (n=11). An outlier is marked as a dot.

The results show a mean acidic mucus cell count of 10.1 (SEM ± 1.6) in [6.75, 14.75] for FTST and 34.4 (SEM ± 4.9) in [22, 39] for CWST. Mean neutral mucus cell count was 0.5 (SEM ± 0.3) in [0, 1.5] for FTST and 2.3 (SEM ± 0.5) in [0, 4] for CWST. There is a significant and large difference in the acidic mucus cells between the field treated and cold-water treated group ($p < 0.05$). There was a noticeable but insignificant difference in neutral mucus cells between the field treated short term group compared the cold-water short-term group ($p > 0.05$).

3.4 qPCR analysis

Outliers in the results are caused by individuals expressing the gene higher or lower compared to the reference point. All the results are shown in tukey box plots made from table 4 and 5 in appendix. Figure 7 shows the relative expression of interleukin 1 β in gills and head kidney samples from the warm-water treated short- (n=8) and long-term (n=7) group. Since the cold-water short-term group was used as base for the calculations of relative expression changes, it is not displayed side by side with the corresponding warm-water group, but included as a control bar.

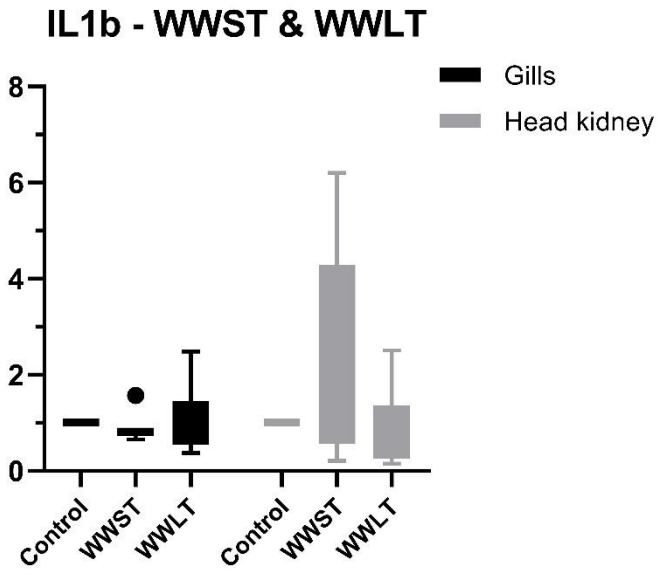

Figure 7: A tukey box plot comparison of interleukin 1β relative expression in gill and head kidney, on a log2 scale, of warm-water short- (n=8) and long-term (n=7) groups. A relative expression value over 1 is a relative increase in expression, while a value below 1 is a relative decrease in expression compared to control group. An outlier is shown as a dot.

The results show a mean decrease in expression of 0.9-fold (SEM ± 0,1) in [0.72, 0.89] in gills from WWST compared to a small increase in mean expression 1.1-fold (SEM ± 0.3) in [0.54, 1.45] in gills from WWLT. There was a mean increase in expression of 2.4-fold (SEM ± 0.8) in [0.55, 4.28] in head kidney from WWST compared to a mean decrease in expression of 0.9-fold (SEM ± 0.3) in [0.25, 1.35] in head kidney from WWLT. There was an outlier in a gill sample from the warm-water short-term group at 1.57-fold increase.

Figure 8 shows the relative expression of interleukin 6 in gills and head kidney samples from the warm-water treated short- (n=8) and long-term (n=7) group. Since the cold-water short-term group was used as base for the calculations of relative expression changes, it is not displayed side by side with the corresponding warm-water group, but included as a control bar.

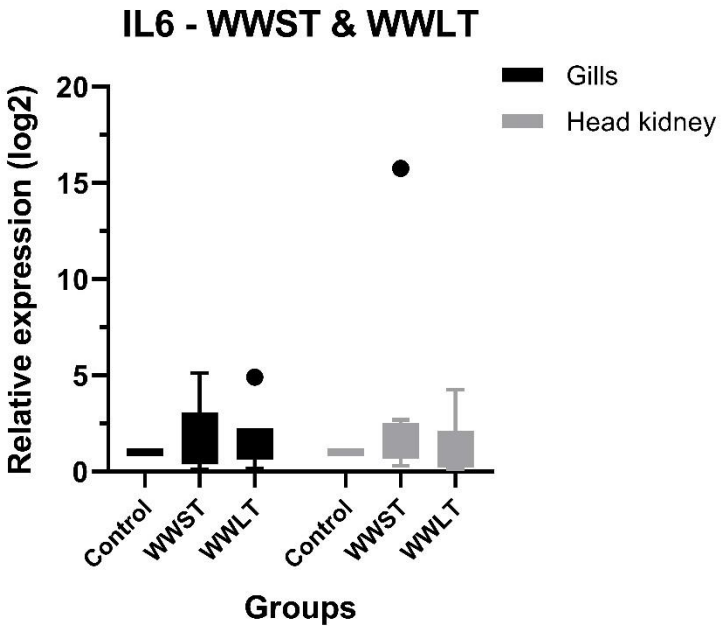

Figure 8: A tukey box plot comparison of interleukin 6 relative expression in gill and head kidney, on a log2 scale, of warm-water short- (n=8) and long-term (n=7) groups. A relative expression value over 1 is a relative increase in expression, while a value below 1 is a relative decrease in expression compared to control group. Outliers are shown as dots.

The results show a mean increase in expression of 1.8-fold (SEM ± 0.6) in [0.37, 3] in gills from WWST compared to a 1.9-fold increase in expression (SEM ± 0.6) in [0.63, 2.25] in gills from WWLT. There was a mean increase in expression of 3.2-fold (SEM ± 1.8) in [0.65, 2.53] in head kidney from WWST compared to a 1.3-fold increase in expression (SEM ± 0.6) in [0.23, 2.13] in head kidney from WWLT. There was an outlier in a gill sample from the warm-water long-term group at 4.9-fold increase and 15.75-fold increase in a head kidney sample.

Figure 9 shows the relative expression of tumour necrosis factor α in gills and head kidney samples from the warm-water treated short- (n=7) and long-term (n=6) group. Since the cold-water short-term group was used as base for the calculations of relative expression changes, it is not displayed side by side with the corresponding warm-water group, but included as a control bar.

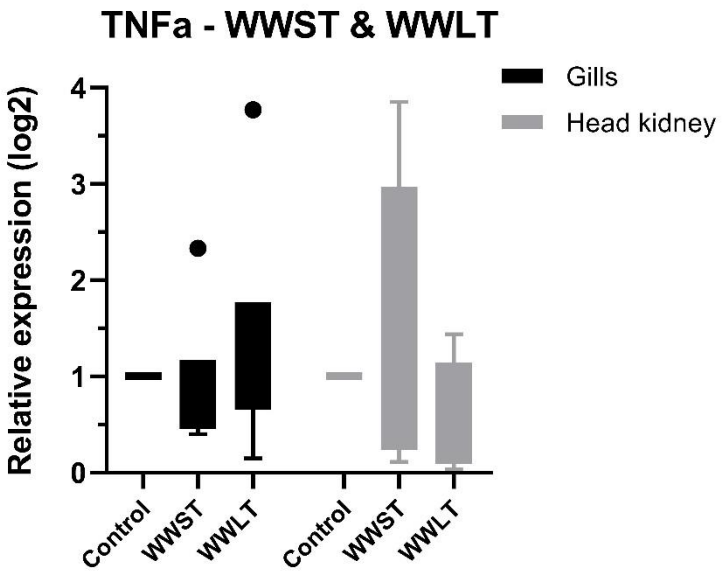

Figure 9: A tukey box plot comparison of tumour necrosis factor α relative expression in gill and head kidney, on a log2 scale, of warm-water short- (n=7) and long-term (n=6) groups. A relative expression value over 1 is a relative increase in expression, while a value below 1 is a relative decrease in expression compared to control group. Outliers are shown as dots.

The results show no difference in expression, meaning a 1.0-fold increase/decrease was found (SEM ± 0.3) in [0.45, 1.17] in gills from WWST compared to a small increase in mean expression 1.3-fold (SEM ± 0.5) in [0.65, 1.76] in gills from WWLT. There was a mean increase in expression of 1.6-fold (SEM ± 0.5) in [0.24, 2.94] in head kidney from WWST compared to a mean decrease in expression of 0.5-fold (SEM ± 0.2) in [0.09, 1.14] in head kidney from WWLT. There was an outlier in a gill sample from the warm-water short-term group at 2.3-fold increase and 3.8-fold increase in a gill sample from the long-term group.

Figure 10 shows the relative expression of heat shock protein 70 in gills and head kidney samples from the warm-water treated short- (n=8) and long-term (n=8) group. Since the cold-water short-term group was used as base for the calculations of relative expression changes, it is not displayed side by side with the corresponding warm-water group, but included as a control bar.

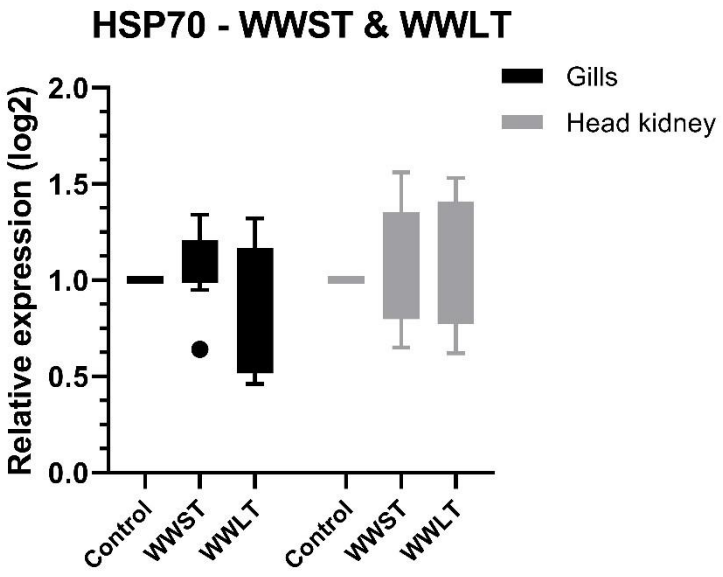

Figure 10: A tukey box plot comparison of heat shock protein 70 relative expression in gill and head kidney, on a log2 scale, of warm-water short- (n=8) and long-term (n=8) groups. A relative expression value over 1 is a relative increase in expression, while a value below 1 is a relative decrease in expression compared to control group. An outlier is shown as a dot.

The results show a mean increase in expression of 1.1-fold (SEM ± 0.01) in [0.98, 1.21] in gills from WWST compared to a 0.9-fold decrease in expression (SEM ± 0.1) in [0.51, 1.16] in gills from WWLT. There was a mean increase in expression of 1.1-fold (SEM ± 0.1) in [0.98, 1.21] in head kidney from WWST compared to a 1.2-fold increase in expression (SEM ± 0.1) in [0.77, 1.41] in head kidney from WWLT. There was an outlier in a gill sample from the warm-water short-term group at 0.64-fold the expression of control.

Figure 11 shows the relative expression of interleukin 1 β in gills and head kidney samples from the field treated short-term (n=8) group. For the field treated group there were no control group taken before the treatment started. Due to this the relative expression values of the field treated group are calculated using the individual with the lowest expression of each gene as a reference value.

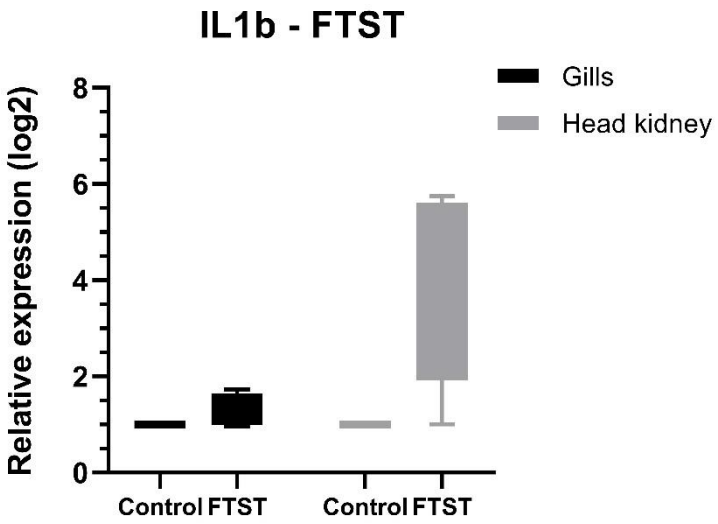

Figure 11: A tukey box plot of interleukin 1 β relative expression in gill and head kidney, on a log2 scale, of field treated short term (n=8) group. A relative expression value over 1 is a relative increase in expression of the gene compared to the lowest expressing individual of that gene from the field group.

The results show a mean increase in expression of 1.3-fold (SEM ± 0.01) in [0.98, 1.64] in gills from FTST compared to the lowest expressing individual for IL1β. There was a mean increase in expression of 3.7-fold (SEM ± 0.7) in [1.92, 5.61] in head kidney from FTST compared to the reference individual. A possible cause of the increased IL1β-expression in the head kidney is later discussed.

Figure 12 shows the relative expression of interleukin 6 in gills and head kidney samples from the field treated short-term (n=8) group. For the field treated group there were no control group taken before the treatment started. Due to this the relative expression values of the field treated group are calculated using the individual with the lowest expression of each gene as a reference value.

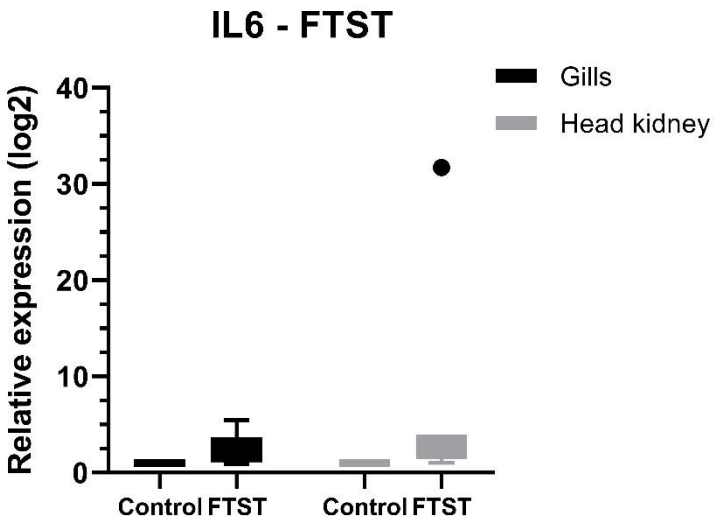

Figure 12: A tukey box plot comparison of interleukin 6 relative expression in gill and head kidney, on a log2 scale, of field treated short term (n=8) group. A relative expression value over 1 is a relative increase in expression of the gene compared to the lowest expressing individual of that gene from the field group.

The results show a mean increase in expression of 2.3-fold (SEM ± 0.6) in [1.09, 3.63] in gills from FTST compared to the lowest expressing individual for IL6. There was a mean increase in expression of 6.3-fold (SEM ± 4.3) in [1.39, 3.90] in head kidney from FTST compared to the reference individual. An outlier in a head kidney sample at 31.71 is marked as a dot and increases the mean and SEM significantly from 2.0 (SEM ± 0.4).

Figure 13 shows the relative expression of tumour necrosis factor α in gills and head kidney samples from the field treated short-term (n=7) group. For the field treated group there were no control group taken before the treatment started. Due to this the relative expression values of the field treated group are calculated using the individual with the lowest expression of each gene as a reference value.

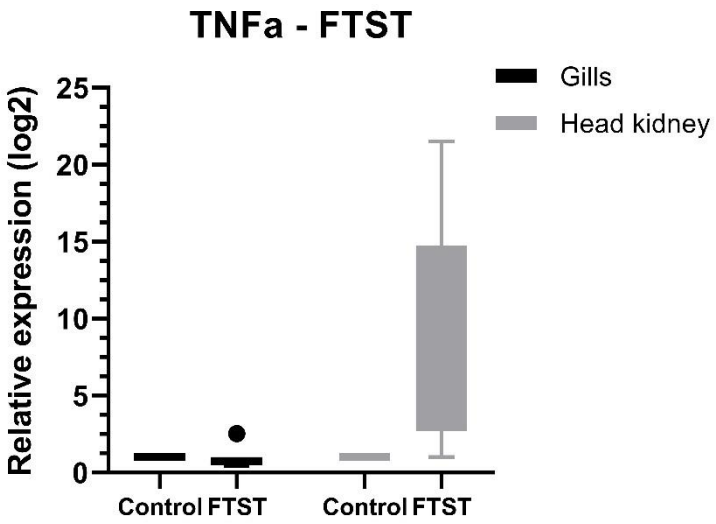

Figure 13: A tukey box plot comparison of tumour necrosis factor α relative expression in gill and head kidney, on a log2 scale, of field treated short term (n=7) group. A relative expression value over 1 is a relative increase in expression of the gene compared to the lowest expressing individual of that gene from the field group. An outlier is marked as a dot.

The results show a mean decrease in expression of 0.9-fold (SEM ± 0.3) in [0.49, 1.0] in gills from FTST compared to the lowest expressing individual for TNFα. There was a mean increase in expression of 9.0-fold (SEM ± 2.7) in [2.71, 14.74] in head kidney from FTST compared to the reference individual. An outlier in a gill sample is shown as a dot on 2.53.

Figure 14 shows the relative expression of heat shock protein 70 in gills and head kidney samples from the field treated short-term (n=7) group. For the field treated group there were no control group taken before the treatment started. Due to this the relative expression values of the field treated group are calculated using the individual with the lowest expression of each gene as a reference value.

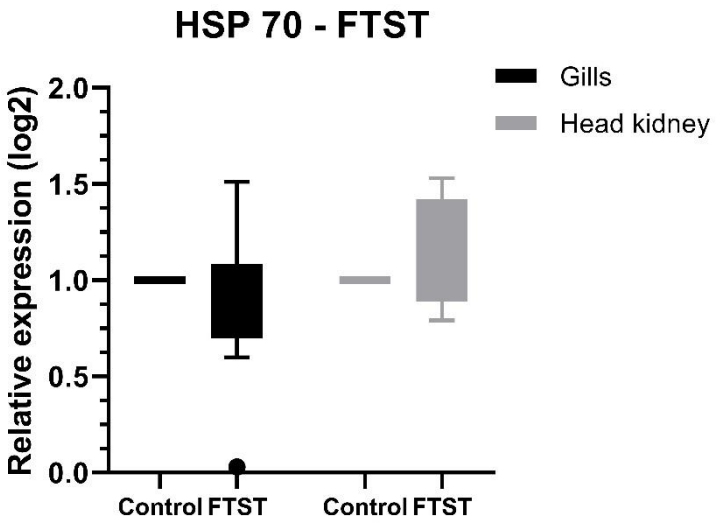

Figure 14: A tukey box plot comparison of heat shock protein 70 in relative expression in gill and head kidney, on a log2 scale, of field treated short term (n=8) group. A relative expression value over 1 is a relative increase in expression of the gene compared to the lowest expressing individual of that gene from the field group. An outlier is marked as a dot.

The results show no change in expression, meaning a 1.0-fold increase/decrease (SEM ± 0.1) in [0.69, 1.1] in gills from FTST compared to the lowest expressing individual for HSP70. There was a mean increase in expression of 1.1-fold (SEM ± 0.01) in [0.89, 1.42] in head kidney from FTST compared to the reference individual. An outlier in a gill sample is shown as a dot on 0.03. This outlier expressed HSP70 significantly lower than the rest of the group, raising suspicion of human error in the qPCR-analysis, see sample 699 in table 6 in appendix.

4. Discussion

The Atlantic salmon fish farming industry's growth has lessened over the last couple of years. Of the 13 zones the coast is divided into, two zones had to stop their growth while another two zones had to reduce their total biomass [114]. The lessened growth is caused by regulatory measurements due to the salmon louse, *Lepeophtheirus salmonis*. When present in large numbers, this ectoparasite can cause damage to the farmed salmon by feeding on the mucus and skin layer, opening it up to infections and osmotic stress. However, the biggest concern is the impact of the spread of lice from farmed salmon to their wild counterpart. This has led to strict regulations laid down by the regulatory authorities demanding delousing treatments when the average number of parasites reaches a fixed limit. Combating this threat is estimated to costing the industry between 4.5 to 5 billion NOK yearly, 2 billion of that is in treatment costs [115].

A Thermolicer treatment uses a 30 second exposure to high temperature water (28-34 °C) to remove salmon louse from the salmon. A single treatment is not always enough, leading to repetitive treatments to completely delouse the salmon. After the treatment there is often a period of increased mortality in the treated sea cages. Examined salmon showed macroscopical injuries to the gills, skin, snout and eyes. Using the welfare indicators from FISHWELL the fish were macroscopically examined before tissue sampling [80]. Histological exams of the gills and skin supported the macroscopical signs, showing aneurisms and lifting of the epithelium in the gills along with scale loss and thinning, or removal, of the epidermis in the skin [85].

The focus in this master thesis has been on the effect of a 30 second 34 °C warm-water treatment compared to a full-scale Thermolicer treatment on Atlantic salmon. Firstly, the 30 second warm-water treatments were done in a controlled setting. This removes the unknown variables found in the field, for instance the crowding time, rough weather and collisions between conspecifics or sea cage equipment. Secondly, two field samplings were done, one during a Thermolicer treatment procedure and one ten days post treatment. This was done to compare full-scale Thermolicer treatments with a controlled warm-water treatment at 34 °C for 30 seconds in controlled laboratory settings. In addition to welfare scoring and histological examinations a qPCR analysis five stress related genes were chosen. These were interleukin 1β,

interleukin 6, tumor necrosis factor α, complement factor C3 and heat shock protein 70. Changes in gene expression is shown as relative expression compared to control by using the ΔΔCt-method.

4.1 Welfare scores

One way to get insight into an individual's state of being is through a set of parameters called welfare indicators. Bleeding in the skin and eyes, loss of scales, wounds and so on are individual welfare indicators. These describe the condition of a single individual, it's not straight forward to extrapolate these indicators to population level, say a population of 200 000 individuals in a typical sea cage. It is therefore important to be critical when looking at a sea cage's mean values of welfare indicator scores [22]. Group based welfare indicators exist, but are focused more on overall mortality, behaviour, surface activity and as such is not an optimal indicator of how the fish is faring immediately after a treatment [80]. Therefore, one must rely on the individual based indicators to estimate how rough an operation like a Thermolicer treatment is on the population.

The mean welfare scores from the controlled and field experiment were meant to give a macroscopical indication of the individuals' condition. In the controlled experiment, mean skin bleeding scores showed no significant differences between either of the warm-water groups compared to their cold-water control groups. There was no significant difference between the mean skin bleeding score of the warm-water short-term and long-term group either. This is despite the long-term group having three weeks to recover from its treatment. As mentioned earlier, superficial wounds heal quickly, depending on the temperature, in Atlantic salmon [87, 116, 117]. Furthermore, it is proved that handling involving handling the fish and/or taking it out of water is a stressor and a cause of abrasions, bruises and scale loss [118-120].

One possible reason for the similar skin bleeding score of both warm-water short-term and long-term groups is that the temperature itself did not cause a macroscopical effect on the skin after the treatment. The skin bleeding that was observed might have been caused by netting the fish

into the treatment box before submerging it into the treatment tank. This is also a possible explanation of the similar skin bleeding scores of cold-water and warm-water groups.

The field group was also scored for skin bleeding: before and immediately after going through the Thermolicer. At both checkpoints, before and after, the mean skin bleeding score was lower than the controlled experiment scores. In theory the field group underwent the same procedures as the controlled ones: crowding, treatment and release. One difference is that the fish from the controlled experiment was netted one at the time, meaning the whole fish was in contact with the net at some point. In the field, moving the fish is done through pumps, this means it is never out of water and there are no nets involved. As stated earlier there is a possibility for collisions between conspecifics and the sea cage walls, but this is a guarantee. A reason for the differences between controlled groups and the field group is that the individuals in the field got lucky. The fish from the field group might not have had any, or few, collisions with conspecifics, nets or walls during the treatment.

In the documentation by Grøntvedt et al. 2015 the effect and welfare of Atlantic salmon going through the Thermolicer machine was tested at four different fish farms, three of these were Atlantic salmon and one was Rainbow trout. The salmon had a mean weight of 2 kg, which puts them between our controlled experiment groups and the field experiment groups. 40 salmons were taken from each fish farm before and after the treatments, as well as at week 1, 2 and 3 post treatment. At each sampling they were welfare scored according to the Veterinary Institute's protocol for welfare scoring [68]. There were no significant skin bleeding score differences before/after the treatment at neither of the three Atlantic salmon farms. Our findings from both the controlled and field experiment agrees with this.

The mean scale loss scores showed no significant changes between the warm-water groups compared to their cold-water control groups. There was not a significant difference between the mean scale loss score of the warm water short-term group and long-term group either. Mean scale loss score in the field was significantly higher after going through the Thermolicer compared to before the treatment. This was also seen in one of the three Atlantic salmon fish

farms the Thermolicer was tested on as part of its documentation [68]. An explanation to this might lie in the method of catching the fish after they have been through the Thermolicer. The way this is done is by pushing a circular capture net below the pipe pumping fish from the wellboat back into the sea cage. The fish is pumped at a decent speed through the pipe and gets dropped around 1.5 meters from the end of the pipe down into the net. This leads to collisions with the capture net wall at quite a high speed, possibly leading to scale loss [118, 120]. This could suggest the scale loss is not necessarily caused by the Thermolicer itself.

Another factor at play might be the size of the fish. The fish in the field study was on average almost twice the size of the fish used in the controlled experiment, and larger weight and larger muscular mass might increase the likelihood of the fish injuring itself while opposing the treatment. The mean weight in Grøntvedt et al. 2015's documentation was 2 kg, placing it in between our own two experiment groups and, in our case, eliminating this factor.

4.2 Histopathological examination

Histopathological examinations were performed to give an indication of possible microscopical changes within the controlled study and field group. The sections were scored on a simple system of 0-3 based on the percentage of tissue affected; 0 (< 5 %), 1 (5-10 %), 2 (10-50 %) and 3 (>50 %) [121]. Of the histopathological changes we observed there were only mild changes in the tissue, where between 5 and 10 % of the tissue was affected, giving it a score of 1.

Thymus is not normally sampled for routine diagnostics in the industry today, but was sampled in our experiment due to its important immunological function in the adaptive cellular immune system (the transport and maturation of T-cells) [122, 123]. The main findings in the thymi examined from both the controlled study and field group were formation of cavities and increase in mucus cells in the thymus tissue beneath the epidermis. Such changes have by some authors been discussed as normal findings, with the degree of occurrence showing normal variations depending on season, life stage and sex [124]. One fish in the warm-water short term exposure group from the controlled experiment had a focal hemosiderosis and a small bleeding. Whether

this single observation can be related to the thermal treatment is uncertain, and it is not uncommon to macroscopically see focal bleedings in the thymus of salmon that has been subjected to stressful events [125].

Mean thymus scores were insignificant in all the controlled experiment groups. The lack of a control group to compare the field Thermolicer group treatment with is a weakness in the study that will be discussed later. However, as an estimate, comparing the thymus score of the field group with the cold-water short-term group acting as control, shows no significant difference. Thymus was not sampled in the documentation study of Grøntvedt et al. 2015 but was sampled in the pilot study of Gismervik et al. from 2019. There were no significant differences between the two studies' examinations of sampled thymus from warm-water treated fish [86].

Gismervik et al. 2019 used a longer exposure time (90-140 s.) and a different scoring system (sparse, moderate, severe) compared to ours (30 s.) and 0-3 [86]. We chose 0-3 like George et al. 2016 since numbers with percentages are less dependent on the readers perspective of what sparse, moderate and severe changes look like. However, their scoring results are translatable and agree with ours when it comes to mean gill scores. In the controlled experiment, the warm water treated short-term group showed a significantly higher gill score compared to the cold-water treated group. However, significant differences, the degree of changes were qualitatively defined as mild. The score differences are mainly based on hyperplasia and light clubbing of the tips of the lamella. One of the fishes from the warm-water short term group had 5 closely grouped aneurisms in an area of the filament but it did not cover enough of an area to warrant a score of 2. It is not possible to prove whether it was caused by the heat or collision with a wall, or whether it represents a sampling artefact. In the long-term groups, there were no differences in gill score between warm- and cold-water treated groups.

Comparing the effect of the Thermolicer field treatment is complicated by the lack of a control group sampled before the treatment began. However, by comparing the Thermolicer field treatment group with the cold-water short-term group as an estimate, no significant difference in mean gill score was seen. This disagrees with the findings of Grøntvedt et al. 2015. In the

three Atlantic salmon farms sampled the gills showed active bleeds, in two of the three farms this bleeding was described as acute [68]. Active bleeds in addition to aneurisms were also found in gill sampled from deceased Thermolicer treated salmon according to Poppe et al. 2018.

As discussed earlier in the welfare section, handling is known to cause damage to the fish. Panic reactions from the warm water could explain the higher mean gill score in the controlled warm-water group. As the fish panics inside of the treatment box, it collides with the walls. This might be a likely cause, seeing how there was no significant difference between the field treated group and the cold-water short-term group. However, this does not explain the difference between the field treated group and the fish farms in the documentation study of Grøntvedt et al. 2015. The Thermolicer has been in active use for five years and has gone through several models of the machine setup, from the prototype testing in 2014 to the Thermolicer System V in 2017 [126]. However, the temperature in the treatment chamber is still between 28-34 °C. A possible explanation might lie in the advancement and experience gained in usage of the Thermolicer procedure. Efficiency in crowding, pumping and treating the fish may affect the possibility of injury the fish is exposed to as well as dedicated wellboats streamlining the process.

Observations in this and other recent studies differs somewhat with the qualitative descriptions given by Grøntvedt et al. 2015 and Poppe et al. 2018. This might stem from the fact that the observations in the two mentioned publications are from earlier versions of the Thermolicer-machine compared to the Thermolicer in this thesis's field group. Thus, it is tempting to suggest that improvement of the equipment and operation procedures may have a role in the lower severity of injuries found in this study, although other factors cannot be excluded.

Pseudobranch is another organ that is not usually sampled [86]. It has an important function in supplying the eyes with oxygenated blood [127]. There were no signs of histological changes in any of the groups except for one small bleeding and one tear in the lamellar tissue, both found in the warm-water short-term group. The tear is likely an artefact from the processing process. Gismervik et al. found no histological changes in their study, even though their exposure time was 2-5 times as long as ours.

The decision to include the pseudobranch was made due to its superficial location and that little is known about how it is affected Thermolicer treatments [85, 127]. Its role is not as immediately life threatening for the fish compared to the gills, but damage to the pseudobranch will be a negative influence on the eyes' blood supply. The Atlantic salmon is a visual hunter, meaning it relies on its vision to hunt for food [128]. Damage to the pseudobranch in Atlantic salmon infected with the parasite *Parvicapsula pseudobranchiola* shows how the fish is affected by pseudobranchial damage. In infected fish, bleeding in the eyes may occur and as the infection progresses this causes a loss of vision or blindness as the oxygen and blood supply diminish over weeks or months [129]. Pseudobranch was not one of the organs examined in the documentation study of Grøntvedt et al. 2015, so comparing how the pseudobranch was affected by Thermolicer treatments in 2015 compared to 2020 is not possible.

Nostrils, eyes and kidneys were also sampled as mentioned in earlier. The nostrils were examined by Gismervik et al. 2019, but at 34 °C and at 2-5 times the exposure time compared to our study there was only seen histopathological changes in one fish. They note that the nostrils are not usually sampled, meaning the interpretation of the results should be done carefully as the results might be caused by artefacts from the processing. Inexperience with the processing and sectioning of nostrils in our experiment made it difficult to get meaningful results. The sections made were often full of artefacts. It was often difficult to make a proper section due to the cartilage around the nasal cavity. After experimenting with different processing and trimming of the nasal cavity it was decided to not continue sectioning to focus our efforts on the organs we had success in sectioning.

Sectioning the large eyes was a challenge. The marble size of the eyes meant they had to be trimmed into a processable size. This was probably the step where artefacts such as tears and crumbling were made. In 4 out of 6 sections made to experiment with the processing method the cornea was crumbled or teared, likely due to poor processing skills. Gismervik et al. 2019 found epithelial changes in the cornea of one of the salmons treated at 34 °C after a longer treatment time.

The kidneys were sampled since we were already sampling the head kidney for qPCR-analyses. Gismervik et al. 2019 found no histopathological changes in the kidneys at the same temperature and longer treatment time. Due to this the kidneys were not prioritized for sectioning. Another thing to note is that due to the large size of the salmon there is a lag before the body temperature starts to increase after being submerged in warmer water [130]. In other words, the 30 seconds treatment time might not influence the internal organs, which agrees with the results of Gismervik et al. 2019.

Fry 1971 saw a difference in the thermal tolerance between the genders of guppies [108]. We gendered the fish sampled in our experiment, but there was no difference between the genders in the histopathological examinations of thymus, gills or pseudobranch.

The lack of a control group for the field short-term is a critical weakness when it comes to comparing and concluding results. We were given a two-hour notice before we had to leave if we wanted to make it to the fish farm before the Thermolicer treatment was scheduled to begin. When we arrived at the fish farm the delousing was underway making it impossible to get both a control group and a treated group, even though there were enough equipment to sample both. There was only one pair of hands available, so the option was to either go to another un-treated sea cage to sample control groups or stay with the Thermolicer to get samples from treated fish. Additionally, FTLT was not from the same fish farm as FTST and was sampled mostly because we got access to a population of fish that had undergone a Thermolicer treatment and was curious about how it looked 10 days later. We followed the method of Grøntvedt et al. 2015, but our results disagree with the acute bleedings they found. The treatment temperature used in the documentation is not mentioned, it might have been higher than the 29 °C used in our treatment.

4.3 Mucus cells

Mucus cells, or goblet cells, are a type of cell producing the mucus layer covering the epithelial surfaces of the fish. The mucus cells can produce glycoproteins of different acidity; some are acidic, and some are neutral. Which acidity the glycoproteins have are produced have been shown to change under stressing and environmental factors [84]. The mucus layer's function as an antimicrobial barrier have however been known for years and a change to this system might expose the fish to possible infections [131].

There are two major glycoprotein components in the mucus [83, 132] amongst other minor components [133]. The glycoproteins' role in the mucus layer is not fully understood, but they are known to act as agglutination factors [132]. Based on this we wanted to look for changes before and after warm-water treatments. Using AB-PAS staining the acidic cells will be coloured a deep blue colour compared to the red/magenta colouring of the neutral cells. How changes in the epidermal mucus cells affects the fish is an area that needs further study [134].

We examined sections of skin and gills, following the method of T. C. Fletcher et al. 1976 who looked at Rainbow trout and Karlsen et al. 2018 who looked at Atlantic salmon. In the skin sections from CWST and WWST there were no significant changes in mucus acidity, i.e. no significant change from acidic to neutral. It was only possible to get sections of skin properly sectioned from 3 fish in the CWLT group for reasons explained earlier. Comparing WWLT to CWST showed no significant differences.

A weakness in the sampling, as mentioned earlier and discussed later, was no control group sampling before the Thermolicer treatment. Therefore, FTST is compared to CWST from the controlled experiment. There was a significant difference in the number of neutral mucus cells from sections of skin. The number of neutral mucus cells in FTST was significantly lower than in CWST. Since there is no control group it is not possible to determine if this was caused by the Thermolicer or if there was no change between before/after the treatment.

In the gill sections from the controlled experiment there were no significant differences between CWLT and WWLT in neither of the mucus cell types. There were no significant differences between CWST and CWLT, nor between WWST and WWLT. In the field experiment there was a significant difference between FTST and CWST. Same as with the skin cells, this might not have been a drastic change compared to before the Thermolicer treatment, but there is no control group to compare this to.

There is, to the best of our knowledge, a lack of studies on the effect of acute thermal stress/Thermolicer treatments on the glycoproteins in mucus cells. Handling, stress and environmental factors affect the mucus layer as discussed earlier, but not how it effects the cells directly. The importance of the mucus layer for the welfare and survival of the fish is perhaps overlooked in the welfare scoring after Thermolicer treatments as it is not easily visible to see a damaged mucus layer.

If the experiment was to be repeated it should include a proper control group for FTST as discussed previously, and FTLT should either be sampled properly with a control group or not be included at all. Due to the circumstances regarding Covid19 the mucus cell counts were not blinded, but it was repeated by the same person to check for differences. There is software available that can assist in the counting of mucus cells by filtering colours in an image. This is helpful in situations with a mix of acidic and neutral glycoproteins inside the same cell. In most of the skin and gill sections the colours were easy to distinguish and count, so no software was employed in this experiment.

4.4 Changes in gene expression

After an exposure to a stressful event the gene expression within the cells of the body alters to better adjust to the situation. In the controlled experiment the control group samples were taken after the fish had undergone a cold-water treatment. Since handling is a known stressor, and most of the genes' expression changes under stress, there might be no discernible difference between the warm-water and cold-water treated fish. In the field experiment the samples were taken before and after a Thermolicer treatment. All samples were stored at RNAlater at -20 °C. The protocols from the two kits, RNeasy 250 mini-kit (Qiagen) and Quantitect Reverse Transcription-kit (Qiagen), was followed. These kits were chosen for their ease of use, previous experience with and their reliability vs. cost per sample ratio. We used a qPCR-analysis of samples from before and after either a 30 second 34 °C warm-water treatment or full-scale Thermolicer treatment. The changes in relative gene expression compared to the control group was quantified using the ΔΔCt-method.

Interleukin 1 β is an important part of the first reaction to stress, and as such it was hypothesized that there would be an increase in its expression after a warm-water treatment. An important point to mention is that the field group's relative expression was calculated by using the individual with the lowest expression of each gene as a reference point. In a study by Fast et al. 2008 on Atlantic salmon, IL1β was significantly upregulated at 1 and 3 hours after a stressing event, but not at 0h or 24h after [92]. We euthanised the fish shortly (> 10min) after the warm-water treatment, and shortly after the Thermolicer treatment. This could mean we missed the sampling window and should have delayed the sampling. In the field group the mean of the head kidney's relative expression of IL1β looked almost twice as high as in WWST. This could look like a high difference between the two experiment groups, but it could likely be a result of no control group for the field group. However, there is an external factor that could in fact make this a real difference in expression of IL1β, even if you cannot directly compare the controlled experiment and the field group.

An infection with *Lepeophtheirus salmonis* has been shown to increase the expression of IL1β in head kidneys of Atlantic salmon 21 days post infection. The field group underwent a

Thermolicer due to the fish farm's high mean mature female salmon louse in the weeks leading up to the treatment. This means an increased relative gene expression of IL1β in the field group is not necessarily caused by the Thermolicer treatment, nor is it necessarily merely a result of what the calculations are based on.

Changes in the expression of interleukin 6 has been seen after increased physical activity in humans. Atlantic salmon in both the 30 second 34 °C and full-scale Thermolicer treatments are exposed to a highly stressful physiological event, so the expression of IL6 was hypothesised to increase after the treatment. Nielsen et al. 1996 saw a rapid increase in IL6 following physical activity in humans, but this was thought to be due to a decrease in plasma volume [100, 135, 136]. This could explain the differences in the spread of relative expression, as the fish is actively trying to avoid its environment as seen by Elliott 1991 on salmonids [137].

Another reason to believe that the differences are not caused by changes in expression is that the expression of IL6 increases after 45-120 minutes post-stress in humans according to Steptoe et al. 2007. This points to delaying the sampling of organs for qPCR-analysis to hit the expression window. The hypothesis that because IL6 has an expression window of 45-120 min post treatment has a flaw in it; humans have a higher core temperature which alters the pharmacokinetics [138]. In gilt-head seabream (*Sparus aurata*) and zebrafish (*Danio rerio*) the expression of interleukin 6 has its peak between 180-360 minutes post stress [139, 140].

Tumor necrosis factor α has several overlapping functions with IL1β. This means it should have an early expression to react to potential infections and to act pro-inflammatory. In a meta study on humans it was found that the concentration of TNFα increased 31-50 minutes after a stressor was encountered, but not later than 50 minutes [141]. An *in vitro* study on six cell lines from Rainbow trout by Hong et al. 2013 showed an increase in the gene expression of TNFα between 15-30 minutes after applied stress in the form of a bacterial infection. This makes it plausible that we may have hit the sampling window in our field group. If the Thermolicer treatment itself was not causing an increased expression, then another possible reason for an increased gene expression may be the salmon louse [142]. In Fast et al. 2006 the gene

expression of TNFα in head kidney was significantly higher 9 days post-infection compared to uninfected Atlantic salmon in their control group, which agrees with our results.

Heat shock protein 70 is constitutively expressed in cells due to its important function in maintaining the homeostasis and assisting in protein folding. Its expression is upregulated by temperature changes, meaning there should be a noticeable increase in expression following the warm water/Thermolicer treatments. Logan et al. 2011 in a study on cooper (*Gillichthys mirabilis*) found there to be a significant upregulation of HSP70 regardless of the fish's acclimation temperature. The Atlantic salmon lives in a colder climate than coopers, this could affect the pharmacokinetics of when the gene increases/decreases in expression. In the controlled experiment groups this was not seen, nor was it seen in the field group, where the lowest HSP70 expressing individual was within a 1-fold increase of the highest expressing one. Either we sampled too early for the expression changes to kick in, or our results disagree with Tomanek et al. 2010 and Logan et al. 2011, which is less likely than us missing the sampling window. Logan et al. 2011 did the first sampling after one hour of heat stress, supporting the hypothesis of us missing the window by sampling less than 10 minutes after the heat stress.

A point to mention is that the whole operation is stressful; the crowding, pumping and general handling of the fish is a stressor. This means that the changes in gene expression might very well have started by the time the fish was sent through the Thermolicer and sampled afterwards. If we had a control group of fish that was sampled earlier in the day, before the whole delousing process began, we could have tested this hypothesis.

The genes chosen in the experiment were picked out after we had done the sampling, which means that by the time we read the relevant literature regarding when the increased expression is seen, we had already sampled. If the experiment were to be repeated one should take this into consideration. However, when planning *in vivo* experiments, what is ideal for the gene we want to examine might not be practically doable. Adding four more genes to the mix makes it near practically impossible to sample once and hit all the hypothetical sampling windows. This is

especially true for conditions like those in the field treatment where we had no say in how quickly the treatment was completed.

There are several reasons for the lack of statistical analysis of the qPCR-analysis. Firstly, the costs of the qPCR-analysis lowered the number of samples analysed leading to a high standard deviation due to the low n (n = 6-8). However, it is normal to run qPCR and statistical analyses like ANOVA on a low n as seen in other studies on Atlantic salmon [143, 144] . Secondly, in a study on the gene expression within the same population of the teleost genus *Fundulus* there was often up to a 2-fold difference between individuals [145]. The samples within the controlled and field experiment were quite spread, as shown in the results. Due to these factors there was not done any statistical analysis on the relative expression data due to the high probability for error.

There is less value in comparing the differences in relative expression between the experiment groups compared to what the lack of differences may tell us. The differences we found can to a degree be explained by the natural spread of the gene expression within a population. Since there is a natural spread in the expression of genes, there are individuals who respond to treatment with stronger or weaker gene expression than others. This could have had an effect during the sampling. We may have sampled fish that were all expressing a gene strongly in one group compared to a mixed group of strong and weak expression in another group, giving us a larger spread. If we take away these effects, what does the lack of clear changes in the expression tell us? Ignoring the debate about different perspectives of what is a clear change and not, what does the gene expressions we found, coupled with our histopathological findings, say?

We planned this experiment with thoughts of how we were going to find massive aneurisms like those from the diagnostical laboratory's report, acute bleedings filling the gill cavity like those found in the documentation from 2015. Based on these assumptions we expected to find clear indicators of stress; expression changes that could not be explained by randomness or individual spread of expression, but rather caused by the immune system reacting to what, in

2015, was proved to be a harsh treatment. Our qPCR-analysis supports our histological examinations in the claim that there was no significant change before and after treatment in either of our experiments within the limitations set by sampling sizes, methods used and (lack of) control groups.

4.5 Future work

A future study should continue work on the repetitive treatments with proper control groups in the field. The welfare documentation of Grøntvedt et al.'s 2015 main concern regarding the Thermolicer was the severe gill injures seen in all four fish farms. In our field study we found no significant gill changes compared to our cold-water treated control group. A larger sample size is needed to conclude that there has been a significant improvement in fish welfare since 2015. Another area worth looking into is the minimum recuperation time needed at a given temperature to make sure treatments are scheduled far enough apart to avoid overwhelming the salmon's ability to recover. In our experiment we did not manage to quantify the effect of heat itself and how it plays a role on warm-water treatments of Atlantic salmon, like a Thermolicer delousing treatment. Improvements and changes to the experiment setup and methods should be considered.

5. Conclusion

There were no significant changes in mean skin bleeding and scale loss scores of the groups in the controlled experiment. We did not find any significant difference between the 34 °C treated and full-scale Thermolicer treated fish regarding skin bleeding score. The scale loss was significantly higher after the Thermolicer treatment. Both results agree with the findings of Grøntvedt et al. 2015. However, these changes in scale loss might have been caused by the method of catching the fish after the treatment.

The histopathological examinations showed no significant changes in samples of thymus and pseudobranchs from all groups. In the gills there was a small, significant difference between the warm- and cold-water treated short-term group seen as hyperplasia and/or bleeding. There was no significant difference from the cold-water short-term group compared to Thermolicer treated group, raising doubts about the results from the warm-water short-term group being caused by the temperature alone.

In the mucus cell counts and acidity in the skin we found no significant differences in the controlled experiment groups. In the field there was a significantly lower number of cells in total compared to the controlled experiment groups. In the gills there were no significant differences in count nor acidity between any of the controlled experiment groups. There was a significant difference in total mucus cell count in gills between field and controlled experiment group.

The qPCR-analyses showed a wide spread in the expression of genes amongst the individuals in each group. IL1β and TNFα was stronger expressed in head kidney of both warm-water treated and field treated fish compared to control/reference fish. In the warm-water treated fish this is suspected to be cause by individual differences within a populations. In the field treated fish, the salmon louse may have had an effect in the increased expression of IL1β and TNFα in addition to the individual variation.

6. References

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Appendix I – Reagents

Reagents	Concentration/content
Alcian blue solution pH 2,5 (Merck)	
Bluing	75 µl NH_3 + 225 ml dH_2O.
Decalc decalcifying fluid (Histolab)	
Ethanol (VWR Chemicals)	96 % & absolute EtOH.
Eukitt (Merck)	
Fast Sybr Green (Applied Biosystems)	
Formalin	100 ml 37 % formaldehyde + 900 ml PBS.
Gill's hematoxylin III solution (Merck)	
Heat shock protein 70 fwr/rev primer (	
Histoclear (National Diagnostics)	
Histowax (Histolab)	
Hydrochloric acid (Merck)	300 µl + 225 ml 70 % EtOH
Periodic acid 0,5 % (Merck)	
Quantitect Reverse Transcription-kit (Qiagen)	
RNeasy mini-kit 250 (Qiagen)	
Schiff's reagent solution (Merck)	
Shandon Instant Eosin (Y) (Thermo Scientific)	
Tricaine (Pharmaq)	

Appendix II – Data material

Table 1: Summarized raw data from the mucus cell typing and count from skin sections.

Group/mucus colour	Acidic mucus cells	SD	Total	Neutral mucus cells	SD	Total
CWT day 1 (n=15)	27,93	21,79	419	20,5	23,86	262
WWT day 1 (n=11)	15,27	17,22	168	22,45	20,76	247
CWT day 21 (n=5)	1,67	2,36	5	35,67	7,76	107
WWT day 21 (n=10)	18,8	19,72	188	24,5	23,28	245
FT day 0 (n=8)	20,88	9,28	167	4,25	4,95	34
FT day 10 (n=2)	33,5	2,5	67	10	2	29

Table 2: Summarized raw data from the mucus cell typing and count from gill sections.

Group/mucus colour	Acidic mucus cells	SD	Total	Neutral mucus cells	SD	Total
CWT day 1 (n=11)	34,45	15,44	379	2,27	1,65	25
WWT day 1 (n=16)	27,06	16,92	433	7	13,15	113
CWT day 21 (n=5)	21	3,29	105	0,8	1,17	4
WWT day 21 (n=9)	22,89	5,34	206	2	1,33	18
FT day 0 (n=10)	10,13	4,28	81	0,5	0,86	4
FT day 10 (n=2)	3,6	3,77	36	0,3	0,9	3

Appendix III – qPCR data

Table 3: Overview of primers used in the qPCR-analysis. Due to circumstances regarding the covid19-outbreak the IL6 effectiveness was not calculated.

Gene	Primers 5'->3'	Acc. nr.	Effectiveness	Publication
ssEF1a Fw	TGCCCCTCCAGGATGTCTAC	BG933853	2,16	Link
ssEF1a Rev	CACGGCCCACAGGTACTG	BG933853	2,16	
ssC3-1 Fw	TCCCTGGTGGTCACCAGTACAC	BI468074	1,95	Link
ssC3-1 Rev	ATGATGGTGGACTGTGTGGATC	BI468074	1,95	
ssTNF alpha 2 Fw	TGCTGGCAATGCAAAAGTAG	AY848945	1,7	Link
ssTNF alpha 2 Rev	AGCCTGGCTGTAAACGAAGA	AY848945	1,7	
ssHSP70 Fw	CCCCTGTCCCTGGGTATTG	BG933934	2,05	Link
ssHSP70 Rev	CACCAGGCTGGTTGTCTGAGT	BG933934	2,05	
ssIL-6 Fw	CGAGTGTACCAGCTTCTTCTT	XM_014143031	Unknown	
ssIL-6 Rev	GGTCTTTGACCAGCCCTATC	XM_014143031	Unknown	
ssIL-1b1 Fw	GCTGGAGAGTGCTGTGGAAGA	XM_014170479	1,92	Link
ssIL-1b1 Rev	TGCTTCCCTCCTGCTCGTAG	XM_014170479	1,92	

Table 4: Overview of the CT-values of all genes and groups from gill samples.

Gill	CWST		WWST		CWLT		WWLT		FTST	
Gen	Spread	Avg.	Spread	Avg.	Spread	Avg.	Spread	Avg.	Spread	Avg.
EF1-α	18,36-22,91	20,53	17,75-24,56	20,39	18,03-20,95	19,47	18,4-25,19	21,71	18,81-20,90	19,95
IL1β	27,28-34,70	30,52	27,79-34,72	30,59	26,73-31,43	28,16	26,01-33,58	29,9	26,29-28-40	27,05
IL6	27,81-35,23	31,19	28,89-34,43	31,01	27,66-33,28	30,73	30,83-36,16	33,01	32,86-37,05	34,85
TNFα	28,71-36,77	31,37	28,95-36,54	31,34	28,19-34,42	29,99	27,32-33,66	30,85	29,21-31,86	30,39
HSP70	19,63-25,40	22,26	19,19-25,50	22,02	19,00-23,72	20,73	19,28-27,47	23,05	19,45-30,24	21,35

Table 5: Overview of the CT-values of all genes and groups from head kidney samples.

Head kidney	CWST		WWST		CWLT		WWLT		FTST	
Gen	Spread	Avg.	Spread	Avg.	Spread	Avg.	Spread	Avg.	Spread	Avg.
EF1-α	18,37-22,59	20,21	18,50-21,51	20,28	20,38-28,15	21,79	19,77-24,25	21,42	17,26-19,86	18,92
IL1β	26,81-35,60	30,38	26,96-35,33	30,56	29,98-37,08	32,22	28,79-34,42	31,73	28,44-32,74	29,64
IL6	30,64-35,32	33,43	29,28-34,23	32,7	28,47-37,29	31,09	30,23-33,64	31,82	31,61-36,87	34,14
TNFα	31,91-34,75	32,84	30,77-38,22	33,83	30,95-34,56	32,58	31,25-37,03	35,19	29,99-35,79	31,58
HSP70	19,63-25,87	21,79	19,95-23,46	21,78	22,02-30,51	24,19	21,00-26,60	23,048	18,95-20,45	19,43

Table 6: List of all qPCR-samples. Samples marked with * had too low RNA-concentration to synthesize cDNA from with the amount of isolated RNA available.

Sample	Group	Organ	EF1-α	IL1β	IL6	TNFα	HSP70
510	WWLT	Gill	18,415	26,611	32,230	28,932	19,309
511	CWLT	Gill	18,687	26,868	30,751	29,090	19,757
512	WWLT	Gill	20,057	29,303	32,015	30,895	21,246
513	CWLT	Gill	18,046	27,054	27,940	28,462	19,073
514	WWLT	Gill	20,744	30,353	31,143	31,161	21,789
515	WWLT	Gill	22,140	30,416	32,268	32,798	23,298
516	WWLT	Gill	23,613	33,123	32,615	36,888	26,044
517	CWLT	Gill	19,664	27,143	33,209	29,340	20,634
518	WWLT	Gill	18,850	26,268	34,512	27,658	20,061
519	WWLT	Gill	23,090	33,238	34,169	N. detected	25,293
520	WWLT	Gill	25,166	N. detected	35,292	N. detected	27,433
521	CWLT	Gill	20,878	31,088	31,512	33,108	23,039
522	CWLT	Gill	19,909	28,684	30,263	29,976	21,175
523	WWST	Gill	17,765	27,939	29,628	29,807	19,221
524	WWST	Gill	19,430	29,702	31,543	31,943	20,975
525	CWST	Gill	19,991	30,424	34,394	33,175	21,811
526	CWST	Gill	22,485	32,701	33,832	34,887	25,386
527	WWST	Gill	21,050	28,175	29,009	30,219	20,136
528	WWST	Gill	18,833	32,851	32,171	34,558	24,607
529	CWST	Gill	18,405	27,391	27,994	28,836	19,648
530	CWST	Gill	19,305	29,422	30,525	30,756	20,586
531	WWST	Gill	22,232	32,851	32,171	34,558	24,607
532	CWST	Gill	19,446	28,239	29,188	30,042	20,958
533	CWST	Gill	21,072	31,620	31,168	31,742	22,521
534	WWST	Gill	23,973	34,462	33,608	N. detected	25,507
535	WWST	Gill	21,331	31,661	29,629	31,291	22,917
536	CWST	Gill	20,671	30,392	29,822	30,184	21,878
537	CWST	Gill	22,905	34,037	32,618	N. detected	25,297
538	WWST	Gill	18,538	28,763	32,560	29,553	20,339
541	WWLT	H. kidney	19,844	32,867	32,804	34,814	21,001
542	CWLT	H. kidney	20,439	30,778	28,820	31,752	22,032
543	WWLT	H. kidney	20,564	31,144	32,501	35,251	21,900
544	CWLT	H. kidney	21,266	30,253	29,862	31,472	22,698
545	WWLT	H. kidney	21,356	33,413	30,360	32,641	22,859
546	WWLT	H. kidney	19,836	30,856	30,389	36,751	21,115

547	WWLT	H. kidney	24,183	N. detected	32,938	38,624	26,334
548	CWLT	H. kidney	21,180	32,167	28,740	33,665	22,437
549	WWLT	H. kidney	20,105	29,060	31,905	31,836	21,552
550	WWLT	H. kidney	24,103	34,426	31,859	N. detected	26,576
551	*	H. kidney					
552	CWLT	H. kidney	21,102	33,268	36,646	34,328	23,353
553	CWLT	H. kidney	28,147	37,079	37,297	N. detected	30,476
554	WWST	H. kidney	20,673	31,814	35,117	32,558	21,606
555	WWST	H. kidney	20,906	30,877	29,590	33,167	22,027
556	CWST	H. kidney	21,242	32,319	33,121	33,780	22,762
557	CWST	H. kidney	23,370	35,601	34,274	N. detected	25,853
558	WWST	H. kidney	20,188	30,118	29,590	36,092	21,387
559	WWST	H. kidney	18,519	26,725	31,082	31,344	19,972
560	CWST	H. kidney	18,584	28,434	30,896	32,628	19,719
561	CWST	H. kidney	18,668	29,539	34,753	32,766	19,909
562	WWST	H. kidney	21,255	34,336	33,205	38,220	23,460
563	CWST	H. kidney	18,593	29,539	33,420	33,340	19,676
564	CWST	H. kidney	18,423	27,050	33,895	32,127	19,788
565	WWST	H. kidney	21,484	33,415	33,813	N. detected	23,468
566	WWST	H. kidney	19,746	28,323	33,234	34,406	21,417
567	*	H. kidney					
568	CWST	H. kidney	22,577	34,578	34,797	N. detected	24,834
569	WWST	H. kidney	19,529	28,892	34,600	32,651	20,945
690	FTST	H. kidney	18,140	29,660	34,363	33,328	18,965
691	FTST	H. kidney	19,838	29,744	31,660	31,608	19,850
692	FTST	H. kidney	19,374	29,207	35,197	30,135	19,427
693	FTST	H. kidney	17,315	28,464	32,159	30,192	18,273
694	FTST	H. kidney	19,806	32,162	N. detected	32,034	20,433
695	FTST	H. kidney	18,814	29,807	34,324	31,567	19,151
696	FTST	H. kidney	19,423	29,519	36,231	30,728	19,983
697	FTST	H. kidney	18,709	28,565	35,046	32,460	19,412
698	FTST	Gill	18,834	26,384	33,792	30,441	19,516
699	FTST	Gill	19,989	26,964	33,763	30,773	25,861
700	FTST	Gill	19,981	26,885	35,377	30,666	21,508
701	FTST	Gill	19,265	26,781	35,271	30,658	20,061
702	FTST	Gill	19,676	26,402	34,504	30,093	20,330
703	FTST	Gill	20,204	26,898	35,251	29,489	20,966
704	FTST	Gill	20,890	28,376	34,279	31,244	21,671
705	FTST	Gill	20,774	27,783	36,609	29,791	20,961
-RT 510	WWLT	Gill	37,785	N. detected	N. detected	N. detected	36,332

-RT 511	CWLT	Gill	35,630	N. detected	33,731	N. detected	34,048
-RT 523	WWST	Gill	29,929	N. detected	29,166	37,287	29,983
-RT 524	WWST	Gill	27,575	N. detected	26,969	32,487	27,766
-RT 541	WWLT	H. kidney	34,657	N. detected	32,830	39,740	33,843
-RT 542	CWLT	H. kidney	30,403	N. detected	29,259	35,955	29,844
-RT 554	WWST	H. kidney	35,420	N. detected	35,850	N. detected	36,833
-RT 555	WWST	H. kidney	31,616	37,673	29,630	35,995	30,498
-RT 695	FTST	H. kidney	36,518	N. detected	N. detected	N. detected	36,091