Master thesis

University of Bergen, Norway

Welfare and efficiency of warm and cold waterfall (low pressure flushing) as delousing treatment of Atlantic salmon (*Salmo salar*)

For the Fulfilment of the Master of science in Aquaculture and Seafood

By

Malin Wei Stalheim

Department of Biology

University of Bergen

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Abstract

Salmonid aquaculture plays an important role in increasing global fish production and the biggest factor constraining its further growth and development in Norway is salmon lice (*Lepeophtheirus salmonis*). Previous projects have shown that flushing with freshwater or seawater with large volume and moderate pressure may be used as a delousing method on Atlantic salmon *(Salmo salar)*. It is also known that bath treatments with cold and especially warm water have a delousing effect but can be experienced stressful and painful for the fish if the temperature is too high. The current trial was conducted to examine if waterfall treatment (low pressure flushing) against lice could be made more efficient by using cold or warm water in the waterfall, while simultaneously maintaining animal welfare by avoiding temperatures that is known to cause pain and by reducing exposure time for the fish in the treatment water.

The results suggest that the waterfall treatment had no effect on sessile stages, but an overall delousing effect of 40-56 % on preadult lice and 16-35% on adult lice, with no delayed long-term effects. Over 70% of the delousing efficiency seemed to be caused by the waterfall itself, rather than other procedural factors. The temperature of the treatment water had no significant effect, most likely due to short exposure time. Furthermore, the waterfall treatment had a somewhat negative short-term effect on welfare. However, the applicable welfare parameters improved significantly after 14 days, suggesting no long-term consequences. Additionally, there was no considerable negative effect of treatment temperature on overall welfare.

1. Introduction

1.1 Salmon farming in Norwegian aquaculture

With the continuous growth of the human population, the world's food demand increases accordingly. Due to the limited spacial potential of the global agriculture industry and limited expansion availability of freshwater aquaculture, marine-based food resources have gained a greater focus in recent years. However, still only 17 % of the global meat production comes from marine-based food, where wild fisheries accounts for over 80 % (Costello et al. 2020). Food from the sea, in particular fish, are a crucial source of essential micronutrients and fatty acids unfrequently found in terrestrial agriculture (Golden et al. 2016; Kawarazuka and Béné 2010). Considering the limitations of harvesting from wild stocks due to unsustainable fishing (FAO 2018), aquaculture as a marine food source plays an important role for increasing global fish production. Norwegian aquaculture consists mainly of Atlantic salmon (*Salmo salar*) and Rainbow trout *(Oncorhynchus mykiss)* and started in the early 1970s when salmonid smolts were placed in open cages at sea (Tilseth, Hansen, and Møller 1991). This enabled the pioneer farmers to exploit these species' extensive potential through selective breeding of desired traits, such as increased growth, delayed maturity, disease resistance and flesh quality (Gjøen and Bentsen 1997). The Norwegian aquaculture industry has grown significantly ever since and in 2019 the first hand value of Norway's salmon production reached 68 billion NOK (SSB 2020), approximately 6.8 billion Euro.

The industry has, however, been facing a series of challenges regarding environment and sustainability in recent years, constraining its growth and increasing the cost of production. The biggest contributor to the increased production cost is the salmon louse (*Lepeophtheirus salmonis*) (Torrissen et al. 2013; Iversen et al. 2020), which is an urgent problem to solve for further growth of the industry and sustainable development (Jevne and Reitan 2019).

1.2 Salmon lice

The salmon louse is a marine ectoparasitic copepod which live on salmonid hosts. The parasite feed on skin, mucus, blood and underlying tissue of the fish and can cause extensive epidermal damage and mortality if left untreated (Johnson and Albright 1991). Additionally, other damaging effects caused by the louse include osmoregulatory stress (Fjelldal, Hansen, and Karlsen 2020), behavioural changes (Øverli et al. 2014; Bui et al. 2018), and an increased

risk of secondary infections (Mustafa et al. 2000; Finstad et al. 2000) The life cycle of the salmon louse consists of eight developmental stages: two planktonic stages (nauplius I and nauplius II), a copepodid stage where the lice actively attach to the host, two sessile stages (chalimus I, chalimus II) and three mobile stages (preadult I, preadult II and adult) (Johnson and Albright 1991; Hamre et al. 2013). The development of the salmon louse is highly temperature dependent and is significantly faster at temperatures up to 24°C (Samsing et al. 2016; Hamre et al. 2019). Severe osmoregulatory problems first appear after lice have reached preadult stages, and studies have shown that the threshold level for mortality on Atlantic salmon is above 30 preadult lice on post smolt ~40 g (Grimnes and Jakobsen 1996) or ten adult lice on migrating smolt (Hoist et al. 2003).

Salmon lice is also characterized by high fecundity and wide dispersion of offspring due to potential hosts being few and widely dispersed in nature (Brooker, Skern-Mauritzen, and Bron 2018). Intensive fish farms in open sea cages have a high density of potential hosts, facilitating salmon lice infestation and sustaining unnaturally large lice populations (Morton et al. 2004; Ugelvik, Skorping, and Mennerat 2017). This has both ecological and economic consequences. Smolt from wild salmon populations migrate past fish farms on their way to the sea each spring, making them susceptible to infestation from fish farms nearby (Halttunen et al. 2018). The energy demanding smoltification process weakens the salmon's immune system resulting in a higher possibility for infection, diseases, and parasites causing mortality (Maule, Schreck, and Kaattari 1987; Johansson et al. 2016; Fjelldal, Hansen, and Karlsen 2020). Thus, on top of negative consequences on the farmed salmonids themselves, -fish farms are affecting the wild salmon populations negatively by increasing lice infestation pressure and consequently increased mortality (Barrett et al. 2020). The economic impact of salmon lice includes treatment and prevention costs as well as a loss of profit due to reduced growth in biomass (Abolofia, Asche, and Wilen 2017). Lice also have high fecundity and short generation time, making them able to rapidly develop resistance against chemicals used for delousing (Denholm et al. 2002; Aaen et al. 2015; Jensen et al. 2020).

To protect wild salmon stocks and to prevent excessive infection within farm networks, the authorities has set seasonal threshold levels of lice, requiring delousing treatments if exceeded (Kragesteen et al. 2019). Legislation sets the threshold level during most of the year to an average of 0.5 adult female lice per fish, but lowered to on average 0.2 adult female lice per fish in the spring due to the post smolt migration of wild salmonids (Lovdata 2020).

1.3 Lice treatments

Delousing with chemotherapeutants were dominating in Norwegian aquaculture from 2012 to 2015 before mechanical, biological, and thermal methods were introduced to supplement or replace the chemical treatments (Overton et al. 2018; Cerbule and Godfroid 2020). The need for supplementing treatment methods was/is caused by chemical treatments not being environmentally friendly and resistance development in the lice due to a dependence on few medicinal classes (Jensen et al. 2020). A variety of non-chemical treatment methods are available in Norway today, such as different types of bath treatments (freshwater, warm water), laser to kill individual lice, cleaner fish or mechanical treatments by soft brushes and/or high pressure pumps (Jensen et al. 2020). Although these methods are more environmentally safe and are generally more effective than chemical treatments, they often require crowding, pumping and/or handling which can negatively affect fish welfare due to stress and physical damage, thus causing higher mortality (Overton et al. 2018; Jensen et al. 2020).

Thermal delousing with warm water up to 34°C has become the most common intensive treatment method in Norwegian salmon aquaculture with over 70 % of treatments registered as thermal in 2017 and approximately 60 % in 2019 (Overton et al. 2018; Sommerset et al. 2020). The method is based on inactivation and detachment of the lice following a short-term exposure (20-30 sec) to moderately heated water (Grøntvedt et al. 2015). Thermal delousing have an efficiency of 75-100 % on mobile stages, but can also frequently cause poor post-treatment welfare with elevated mortality (Grøntvedt et al. 2015; Overton et al. 2018). Studies show that salmon have a clear pain-like response when exposed to water above 28°C (Nilsson et al. 2019), recent studies, do however, suggest that so long the warm water treatment is short-term, the warm water itself does not cause acute damage to the fish (Moltumyr et al. 2021).

Delousing with cold water is not a commercialized method but have shown to have some effect on lice. According to a study by Overton et al. (2019), fish transferred from 15°C to -1°C for 10 minutes had a 40 % reduction of lice, although not very effective on sessile stages. Cold shock, which is a physiological response to rapid decrease in seawater temperature (Donaldson et al. 2008), was observed during both -1°C and 1°C treatments and may provide an obstacle for this technique. Although cold shock has an immobilizing effect on the fish resulting in uncoordinated swimming patterns and loss of equilibrium, it is highly

dependent on the temperature drop and exposure time to cold water (Donaldson et al. 2008; Foss et al. 2012). As studies by Foss et al. (2012) and Overton et al (2019) suggest, the exposure time and physical stress from handling is of larger significance than the temperature drop on animal welfare.

Mechanical delousing is commercially used in salmon aquaculture and usually consists of crowding before the fish is pumped up in a treatment system where lice is mechanically removed by flushing with pumps and/or soft brushes (Jensen et al. 2020). Previous projects have shown that flushing with freshwater or seawater with large volume and moderate pressure can be used as a delousing method of Atlantic salmon (Torgersen 2016, 2017). The waterfall alone had a delousing efficiency of 40 – 50 % on mobile lice in the first trail (Figure 1.3.1).

Additionally, bath treatment with warm water (30 – 33°C) for 30 seconds prior to the waterfall treatment further amplified the delousing efficiency in the second trial (Torgersen 2017), where the efficiency reached above 90 % on mobile lice (Figure 1.3.1). However, despite the optimization of pressure and nozzles in previous projects with the waterfall, Torgersen (2016) warns that the treatment can cause scale loss, which must be monitored. Thus, combining waterfall and extreme temperatures (temperature shock) may increase its efficiency but it is also important to ensure that the welfare of the salmon is maintained.

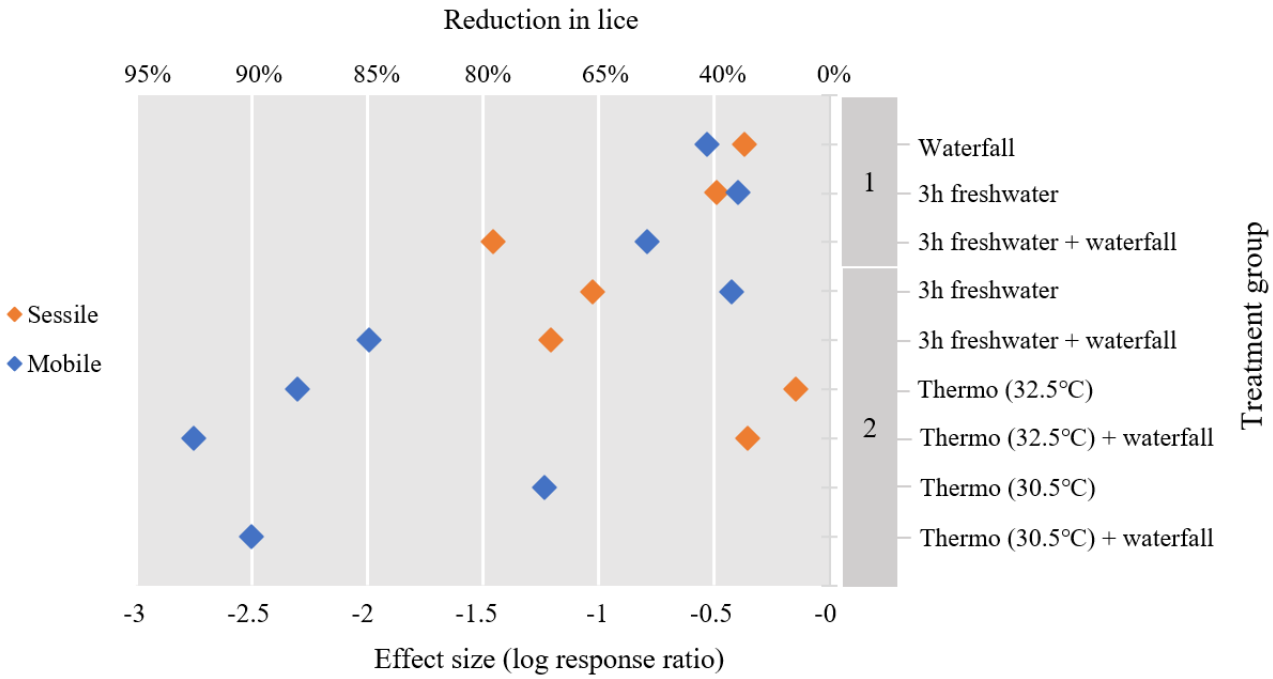


Figure 1.3.1: *Comparison of reported efficiency of delousing with waterfall from two previous trials: 1:Torgersen 2016 and 2:Torgersen 2017. Effect sizes are taken from the previous reports showing relative delousing efficiency between treatments and reference groups, presented as a log response ratio: RR = ln(T/R), where T is the treatment groups and R is the reference groups. Negative effect sizes indicate delousing efficiencies.*

1.4 Objective of the research

The main goal with the trial is to test if the efficiency of mechanical delousing with waterfall can be increased using warm or cold water (27℃ or 0℃ respectively) in the treatment. Subgoals is to investigate the effect of the temperature difference (ΔT) for the fish acclimatized to either of two temperatures (8℃ or 15℃), as well as how the treatment affects welfare.

Despite the optimization of pressure and nozzles in previous projects with the waterfall, Torgersen (2016) warns that the treatment can cause scale loss, which must be monitored. An important part of the experiment is therefore to monitor whether acute damage to the fish occurs during treatment by conducting welfare scoring directly after the procedure in addition to effects on welfare that only become apparent over time.

$H_{1.1}$: Treated groups (0, 27 and 8/15°C) have lower lice levels right after treatment and two weeks after treatment compared to untreated groups.

$H_{1.2}$: Groups treated with 0 or 27°C have higher delousing efficiency than groups treated with the same temperature as the holding water (8°C for block 1 and 15°C for block 2).

$H_{2.1}$: Welfare scores at the end of trial (two weeks after treatment) < welfare scores right after treatment > welfare scores before treatment.

$H_{2.2}$: Treated groups (0, 27 and 8/15°C) have higher welfare scores right after treatment and two weeks after treatment compared to untreated groups.

2. Materials and methods

The experimental work for this thesis was conducted in accordance with the Norwegian laws and regulation on animal experimentation. The experiment was approved by Forsøksdyrutvalget (FOTS identification number 23818).

2.1 Fish and experimental facilities

The trial was conducted in the Tank Environmental Lab (TEL) at Matre research station, Institute of Marine research (IMR), in Masfjorden municipality (Norway) from 03.06.2020 – 13.08.2020. Atlantic salmon post-smolts from IMR's standard production of experimental fish were used in the trial. The fish (n = 800) originated from the Aquagen strain and had a weight of approximately 200 – 400 gram and an age of 19 – 20 months.

The salmon had been produced under standard rearing conditions in increasing tank sizes, following egg delivery in January 2019. This involves using optimum temperatures during egg and fry development, continuous light from first feeding and smoltification initiated by natural short daylength in autumn followed by six weeks of continuous light to finalise parr-smolt transformation. Fish were transferred to full strength seawater during early winter and kept on simulated natural light conditions until used in the experiment.

At the start of the trial 03.06.2020, the experimental fish was moved into two circular tanks (diameter = 3 m, height = 1.25 m, water level 0.5 m, tank volume = 3.5 m^3): tank 4 and 6 in TEL, Hall 4 at IMR Matre. To ensure enough fish by the start of the treatment (n = 800), the tanks were stocked with approximately 450 fish each. The fish in tank 4 (block 2) and 6 (block 1) were held at temperatures of 15℃ and 8℃ respectively. These temperatures were selected based on normal summer/winter temperatures along the Norwegian coast. A lower water level (0.5 m) was held to avoid fish jumping out during lice infestation and when lice moulted from sessile to mobile stages. Both tanks were supplied with filtered, UV treated, aerated saltwater (34‰) from the local fjord at 90 m depth. Heated and chilled water were aerated again prior to each header tank, supplying 100 % air saturated water. The waterflow in the tanks were kept at approximately 100 L/min and were only changed during experimental procedures. Oxygen level was measured continuously and if needed, addition of hyper oxygenated water was supplied, or oxygen was added through diffusors in the tanks and tubs. Oxygen was aimed to always maintain a level above 80 % saturation. The fish were held at a

simulated natural light regime (Light:Dark, 24:0 (June), 19:5 (August) and fed pellets (Skretting, Nutra Supreme 3.0 – 4.0 mm) via an automated feeding system (Arvotec feeding units: Arvo-Tec T drum 2000, www.arvotec.fi) according to standard feeding regimes recommended for their size and temperature, with adjustment based on presence of waste feed on daily observations. Feeding, water flow and temperature were automatically controlled using custom made computer software (SD Matre, Normatic AS, Nordfjordeid, Norway).

2.2 Salmon lice rearing, incubation, and infestation

The salmon lice used in the trial were collected from fish held in rectangular tanks (0.9 m × 0.9 m × 0.5 m, volume = 0.405 m^3) in adjacent tank lab facilities, Hall 4 at IMR Matre. Lice were originally sourced directly from nearby fish farms. The host fishes were held at 12°C, simulated natural photoperiod and standard rearing conditions. Egg strings from lice were collected on 03.06, 10.06, 12.06, 19.06 and 07.07 using the following procedure:

Host fish were sedated in holding tanks of 405 L (water level reduced to 1/3, 15 ml Aquacalm added) before being transferred to a portable tank on wheels with Finquel (100 mg/L) (Veterinærkatalogen 2019). Females were taken off the fish with tweezers and temporarily placed on a tray. One louse at a time were picked up and placed on the hand to easier access the egg strings. Egg strings were collected by carefully pulling them off with tweezers before being put into an incubator. All adult female lice were placed back on the host fish. The fish were transferred to another portable tank with clean water before going back in their respectable holding tanks. After harvesting, the egg strings were incubated at either 11°C or 15°C depending on when they were needed for infestation (Appendix 2). The incubator system (L. A. Hamre, Glover, and Nilsen 2009, Figue 2.2.1) consisted of a hose leading from a header tank into a bucket with water filtration. A smaller tube led the filtered water to the incubator itself, consisting of two rectangular boxes (17.5 cm × 15.5 cm × 13 cm, drain 8.5 cm above bottom, water volume = 2.3 L). The inner box had a filter on the bottom to keep the lice copepodites inside and the outer box had a drain to prevent water overflowing. Up to three incubators at a time sourced water from the same bucket of filtered water.

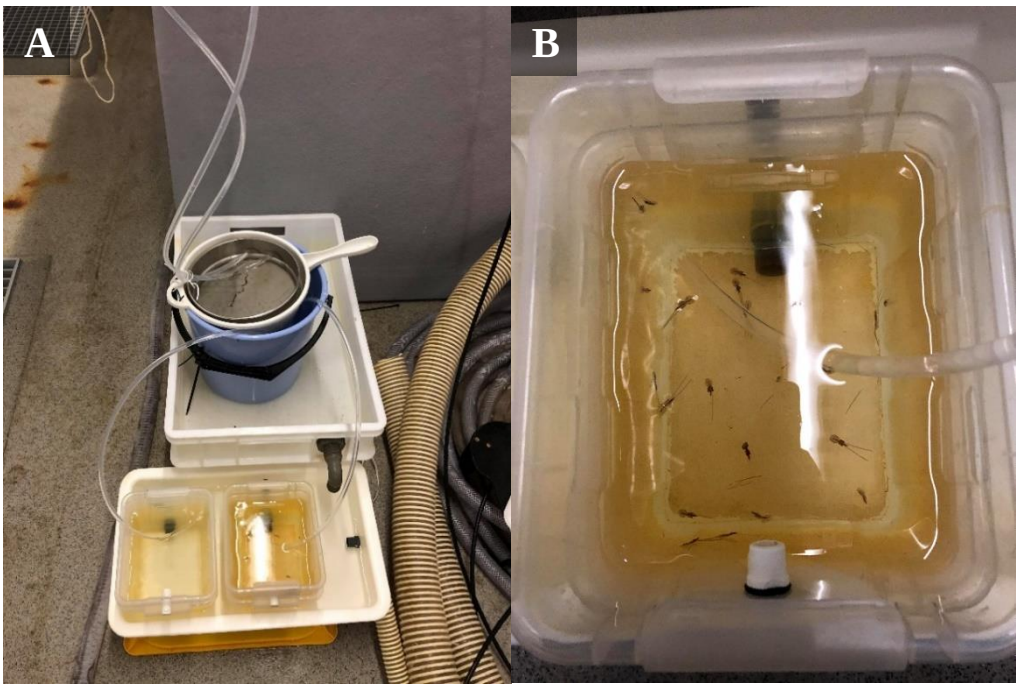


Figure 2.2.1: *Incubator system at either 11℃ or 15℃. A: Two incubators source water from one bucket of filtered water originating a header tank. B: Closeup of one incubator with lice.*

Infestation of the lice started with lowering the water level in the fish tank to approximately 1/3 (~1000 L) before adding the copepodites to the water. The water flow was maintained at 100 L/min, so the water did not reach the outlet until after 30 minutes of the initial infestation. An estimated amount of 4000 copepodites were added to each tank of 400 fish (expected 50 % infestation success) per infestation round (4000 cop × 0.5 / 400 fish = 5 lice per fish).

2.3 Experimental design

The batch of experimental fish (n = 800) were split into two subgroups (n = 400) at the start of the trial and were held on different temperatures (block 1 = 8℃, block 2 = 15℃) in two separate tanks throughout the trial (Figure 2.3.1). Measurements of lice levels and welfare scores were taken at three points of the trial for each block (Figure 2.3.2): pit-tagging 2-4 days before the treatment (B = before), right after the waterfall treatment (A = after) and at the end of the trial, two weeks after treatment (E = end). The treatment groups (n = 100) went through treatment with water temperatures 0℃, 27℃ and 8/15℃ (procedural control for block 1and 2 respectively), before being transferred back in tanks with the initial holding temperature (Figure 2.3.1). Each block also had an untreated procedural control group (n = 100).

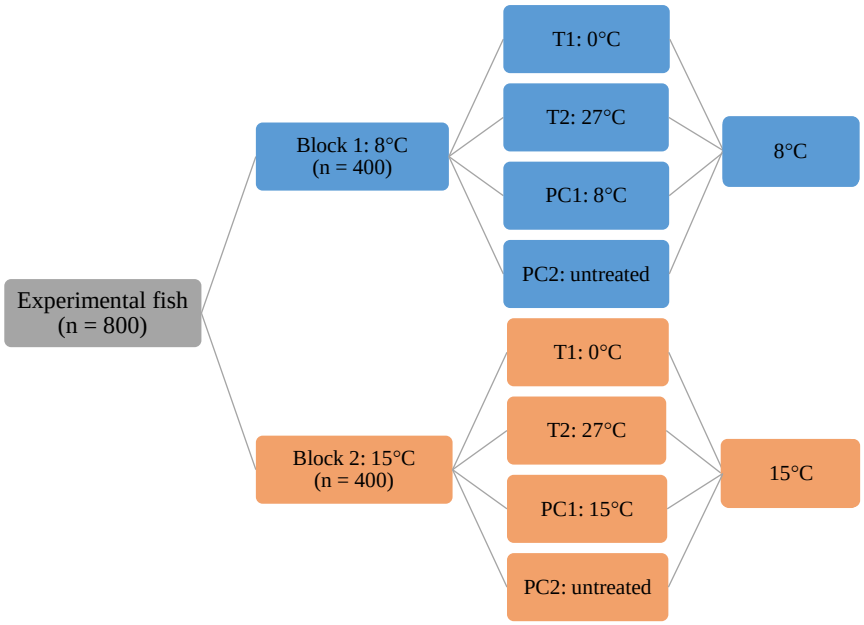


Figure 2.3.1: *Overview of the group setup. Experimental fish (n = 800) were split into two blocks (n = 400) held at different temperatures (block 1: 8°C and block 2: 15°C). Each block had two treatment groups (T1: 0°C and T2:27°C) and two procedural control groups (PC1: 8/15°C and PC2: untreated), with 100 fish per group. After treatment, the fish were held for two weeks at their initial holding temperature for the respective blocks.*

To have an estimated 15 lice in total per fish (5 of each stage chalimus I-II, preadult I-II, adult) at the start of treatment, both temperature groups were infested with salmon lice copepodids three times each (Figure 2.3.2). The fish in both blocks were acclimatized to the holding temperatures of 8°C (block 1) and 15°C (block 2) for eight days prior to the first lice infestation. The time between infestations and treatment was adjusted according to the different temperature groups and known development time of salmon lice using Hamre et al. (2019).

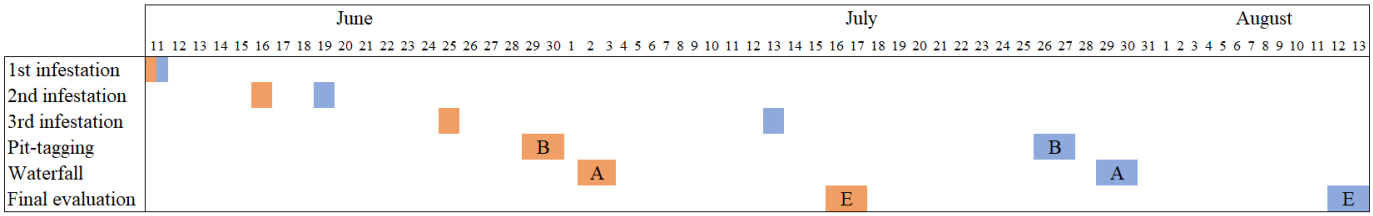


Figure 2.3.2*: Timeline for procedures. Holding temperatures throughout the trial: blue = block 1 (8°C), orange = block 2 (15°C). The experimental fish were infested with lice in three rounds (1st to 3rd infestation). Measurements of lice levels and welfare scores were taken at pit-tagging 2-4 days before the treatment (B = before), right after waterfall treatment (A = after) and at the end of the trial, two weeks after treatment (E = end).*

2.4 Pit tagging procedure

Due to a limited number of tanks being available for the trial, a common garden design was chosen for the experimental setup. Pit tagging the fish made it possible to keep all the fish within the same holding temperature in one tank, despite being from different treatment/control groups. Thus, tagging the fish allowed us to follow the development at an individual level to investigate the long-term effects on lice levels and welfare of the treatment. The pit tagging procedure was conducted 2-4 days prior to treatment to ensure adequate recovery time for the fish before undergoing another procedure, while simultaneously estimating the lice count as accurate as possible. The fish were counted for lice and scored for welfare when tagged, detailed in section 2.3.3 and Appendix 1.

The fish were tagged with one of two standard methods, abdominal (Gries and Letcher 2002; Larsen et al. 2013) or operculum (Biomark 2019). Comparison of these methods displayed no significant differences on performance or welfare and is reported elsewhere (Oldham et al., n.d.). All fish were tagged with the same tag type: 12.5 mm long × 2.12 mm diameter (tag volume = 44.12 mm^3), 106 mg (in air), full duplex (FDX) PIT tags, designed for subcutaneous or intramuscular implantation in animals. Any fish that was accidentally tagged wrongly or did not wake up after tagging was discarded and replaced so that each block consisted of 400 tagged fish by the end of the procedure.

Preparation for the tagging procedure included lowering the water level of the tank to approximately 1000 L as well as reducing the water current to approximately 30 L/min. Oxygen was added in the tank using a diffusor and pressure adjusted to a milky cloud of oxygen was visible in the water under the whole procedure, ensuring adequate level >80 % dissolved oxygen saturation. A calming dose of Finquel was added to the tank (10 mg/L) and supplemented as needed to maintain a light sedation of the fish. One person stood inside the tank with waders and collected five fish at a time with a bucket which were transferred to a portable tank with full anaesthesia (Finquel, 100 mg/L). The fish were then transferred individually to a table where they were tagged by one of the two methods mentioned above, pit scanned for ID, counted for lice, and scored for welfare before they were returned to a new holding tank with the same water quality as the fish were held at prior to tagging.

The tagging procedure lasted two days for both blocks in the trial. For block 2 (29.06 – 30.06), 250 fish were tagged the first day (100 abdomen, 150 operculum) and 150 fish were tagged the second day (all abdomen). For block 1 (26.07 – 27.07), 200 fish were tagged each day. All abdomen tags first day and all operculum tags the second day.

2.5 Waterfall specification and procedure

Waterfall specification

The waterfall consists of a 5.9-meter-long channel with seven circular bars at the bottom, like a sorting grid (Figure 2.5.1). Over the sorting grid there are four horizontal rows of pipes with seven nozzles each, pointing at a 90-degree angle to the bars. The length between the first and last row of nozzles is 2.7 meters, and a manometer is mounted on each of the rows to control the water pressure. The water pressure in the waterfall was set to 0.6 bar, which were checked and noted before every treatment (details in Appendix 3). The waterfall was elevated prior to being used in the trial and adjusted so the slope of the sorting grid was approximately ten degrees to make the fish slide through and end up in a tank (1000 L) at the bottom end. Water was supplied from this tank, filled up to ¾ (~750 L), recirculated but changed in between treatments according to the planned treatment order. The treatments (Figure 2.3.1) per block were randomized within each of four rounds of 25 fish per treatment, making a total of 100 fish per treatment per block (Appendix 3). Ambient water from the fish header tank supplied water temperatures the fish were acclimatized to (8°C or 15°C). Heated water (20°C) was sourced from a separate header tank and further heated using a pool heater (15kW, Electro engineering Ltd). Cold water was obtained by filling a tank with water and chilling it in a refrigerated room (4°C) overnight. Additionally, buckets of ice were made to further chill the water down to 0°C before being used in the waterfall. A bilge pump (Jumbo 50ND, ABSgroup) was placed for tank recirculation before the water was used in the waterfall. The temperature of the supplying water was checked and noted before and after every treatment/procedural control group, as well as air temperature.

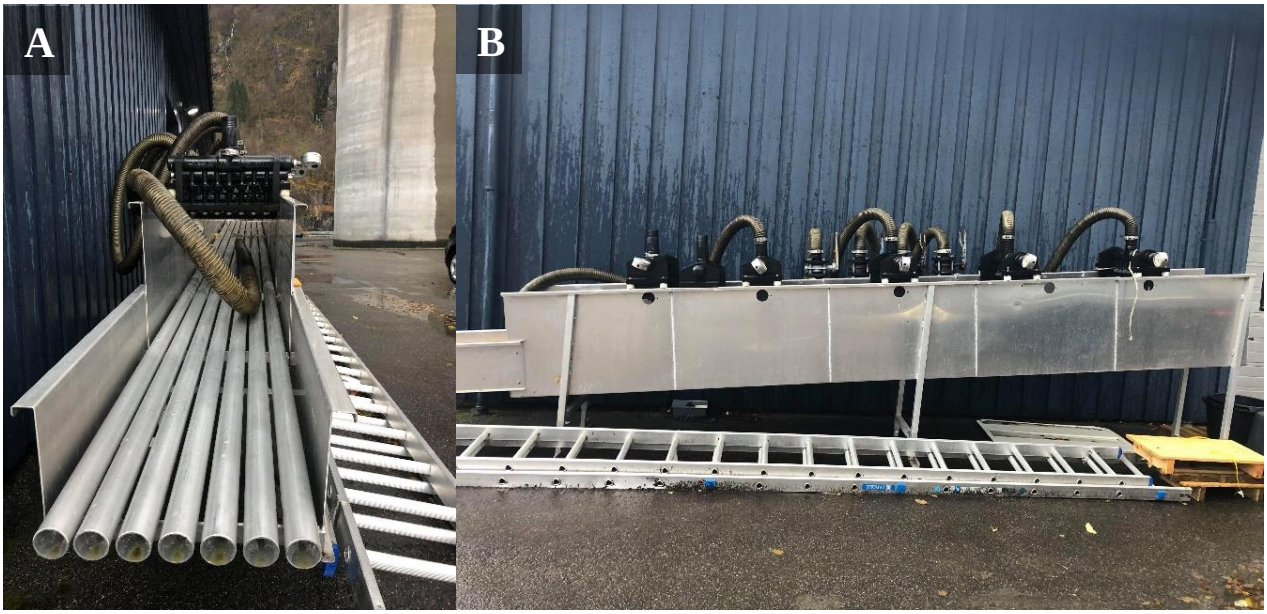


Figure 2.5.1: *The waterfall (was elevated prior to use in the trial). Consists of a 5.9-meter-long channel with seven circular bars at the bottom. Above the bars are four horizontal rows of pipes with seven nozzles each, pointing at a 90-degree angle to the bars. A manometer is mounted on each of the rows to control the water pressure (set to 0.6 bar during treatment). A: Front view. B: Side view.*

Waterfall procedure

The waterfall procedure was conducted over two days (02.06 – 03.06 for block 1 and 29.07 – 30.07 for block 2), going through 200 fish each day. The fish were deloused with one of the treatments or the control treatments as outlined in the group setup (Figure 2.3.1). In essence, after treatment, the fish were anesthetized, scanned for pit-tag, measured for length and weight, counted for lice, and scored for welfare (details about the latter two in section 2.3.3). The fish were then returned to a holding tank and kept for 14 days before once again being anesthetized, scanned for pit-tag, counted for lice, scored for welfare, and euthanized by a blow to the head. Timeline for conducting the procedures is described in Figure 2.3.2.

Since it is well known that handling (crowding, use of nets, etc.) itself has a delousing effect, one group from each acclimated temperature was exposed to the same treatment except for the waterfall. Including the transfer from holding tank to anaesthesia, lice counting, welfare score and transfer back to holding tank. This untreated group is important to give correct figures for the delousing effect of the waterfall at the different temperatures.

Preparations for the waterfall procedure included reducing water flow and lowering the water level in the holding tank, as well as adding oxygenation. The fish were sedated lightly in the holding tank (Finquel, 10 mg/L). One person went into the holding tank and took out five fish at a time with a bucket which were given to person number 2. The second person sent individual fish through the waterfall while person 3 caught them at the other end with a bucket and immediately put them in a portable tank with a calming dose Finquel (10 mg/L). Some fish (~10 %) got stuck in the waterfall, but quickly got a little push from a 4th person on the side with a pole. When the whole treatment/procedural control group (n = 25) was sent through, the portable tank was transferred to a table at the other side of the waterfall. The fish were then individually measured for length and weight, scanned for pit, counted for lice, scored for welfare, transferred to a new holding tank, and held for 14 days after treatment. For the untreated control group of the round, 25 fish were transferred from the holding tank directly to the portable tank with a calming dose Finquel (10 mg/L). Five fish at a time were then transferred by hand to another portable tank with full anaesthesia (Finquel, 100 mg/L) before measurements were taken as described above.

The final evaluation followed the same procedure as just described, subtracting the waterfall and length/weight measurements. Lastly, at the end of the trial, the fish were euthanized by a blow to the head before their pit tag was removed. Also like the previous procedures, the final evaluation lasted for two days for both blocks.

2.6 Lice counting and welfare scoring

Measurements of lice levels and welfare scores were taken at pit-tagging, right after waterfall treatment and at the end of the trial (two weeks after treatment). The counting and scoring were conducted by the same person for all the procedures throughout the trial.

Lice counting

The lice counting was executed in a white, lidless tub filled with enough water to almost cover the fish when put on its side. Lice were counted systematically starting with the ventral side up, rotated to dorsal side to count one side and back to ventral to count the other. The lice were categorized into three stages while being counted: sessile (chalimus I and II), preadult (I and II) and adult. To ensure better accuracy, a headlamp was always used when counting.

Welfare scoring

FISHWELL, a scoring system based on a set of selected welfare indicators (WIs), was used to evaluate the welfare of the fish (Noble et al. 2018). Only morphological welfare indicators for diagnosis and classification of important external injuries were used in the present trial (Appendix 1). The welfare was scored on a scale of 0-3 for all parameters, a lower WI corresponding to better welfare. The welfare parameters chosen for the current trail was focused on the head (snout damage, eye bleeding, protruding eyes, cataract, and operculum damage), skin (scale loss, skin bleeding, acute wounds, and bacterial wounds) and fins of the fish (fin bleeding, fin splitting, and fin erosion). Other abnormalities on the fish observed were also noted during scoring.

2.7 Calculation of delousing efficiency

The results for lice levels were analysed in two ways, using different reference groups for comparison, and were calculated as follows:

I: Control vs. treatment. The first method uses the number of lice on untreated, procedural control groups (n lice control) as reference (Figure 3.1.1):

$$Delousing\ efficiency\ (\%) = \frac{n\ lice\ control - n\ lice\ treatment}{n\ lice\ control * 0.01}$$

II: Before vs. after treatment. The second method uses the number of lice 2-4 before treatment (at pit-tagging) as reference (Figure 3.1.2):

$$Delousing\ efficiency\ (\%) = \frac{n\ lice\ before\ treatment - n\ lice\ after\ treatment}{n\ lice\ before\ treatment * 0.01}$$

2.8 Statistical analyses

Statistical analyses and tests were conducted using R (RStudio version 1.2.1335), including the following packages: tidyverse (Wickham et al. 2019) and gridExtra (Auguie and Antonov 2017). Figures and tables were made in Microsoft Office 365 excel, and R (RStudio version 1.2.1335).

To compare the difference in lice before and after treatment, as well as at the end of the trial, a Wilcoxon test was used investigate if the difference were significant between treatment/control groups. The Wilcoxon rank sum test was chosen as it is nonparametric and can be used on all the data types from the experiment, including count data, differences in lice count before and after treatment, differences in welfare scores before and after and differences in lice levels/welfare scores between groups. Testing several differences before and after increases the risk of getting false rejections of the null hypothesis (Type I error) due to multiple comparisons. A Spearman's rank correlation test was used to evaluate the association between fish size and delousing efficiency. A two-proportions z-test was used to compare proportions (delousing efficiency) between groups after treatment using lice levels on untreated, procedural control groups as reference.

The degree of significance between the groups in this study was considered as significant when p-value < 0.05 and flagged with one star (*). If the p-value is less than 0.01, it is flagged with two stars (**). If a p-value is less than 0.001, it is flagged with three stars (***).

3. Results

Lice- and welfare status before treatment

The time-shifted infestations gave an average level of 1.86 sessile, 0.75 preadult and 4.57 adult lice per fish in block 1 prior to treatment. Block 2 had an average of 0.63 sessile, 5.78 preadult and 1.03 adult lice per fish. Thus, as a starting point before treatment, the fish had an average of 7.18 lice in total for block 1 and 7.44 lice in total for block 2 (Figure 3.1.3).

The welfare levels at start ranged from WI ≤ 1 for snout damage; WI ≤ 2 for eye bleeding and skin bleeding; WI ≤ 3 for scale loss, fin bleeding, fin splitting and fin erosion overall. The other welfare parameters investigated in this study: cataract, protruding eyes, operculum damage and acute/bacterial wounds were generally low, with WI ≤ 1 overall for all groups in both blocks and were consequently not included in the figures below (shown in Appendix 6).

3.1 Lice levels

Control vs. treatment

In the trials of Torgersen (2016, 2017), delousing efficiency was presented as effect size compared to controls, rather than by comparing the same fish before and after treatment as below. Results from the current trial is thus also analysed using the lice levels on the untreated groups on treatment day as reference (Figure 3.1.1), to allow direct comparison with previous data from the "waterfall setup" and to increase the accuracy in delousing effect calculations by removing lice development factors.

Delousing efficiency on sessile lice right after treatment was close to zero for both blocks (Figure 3.1.1) with an average reduction of 7 % for block 1 and 3 % for block 2. On average, the efficiency on adult lice was higher, 35 % for block 1 and 16 % for block 2, and the efficiency on preadult lice was the highest, 56 % for block 1 and 40 % for block 2 (Figure 3.1.1). The total reduction in lice after treatment was similar for both blocks, 27 % and 25 % on average for block 1 and 2, respectively (Figure 3.1.1). The statistical tests on the difference in efficiency between all treatment groups are presented in Appendix 4.
At the end of the trial the total decrease in lice was 19 % for block 1 and 16 % for block 2 (Figure 3.1.1).

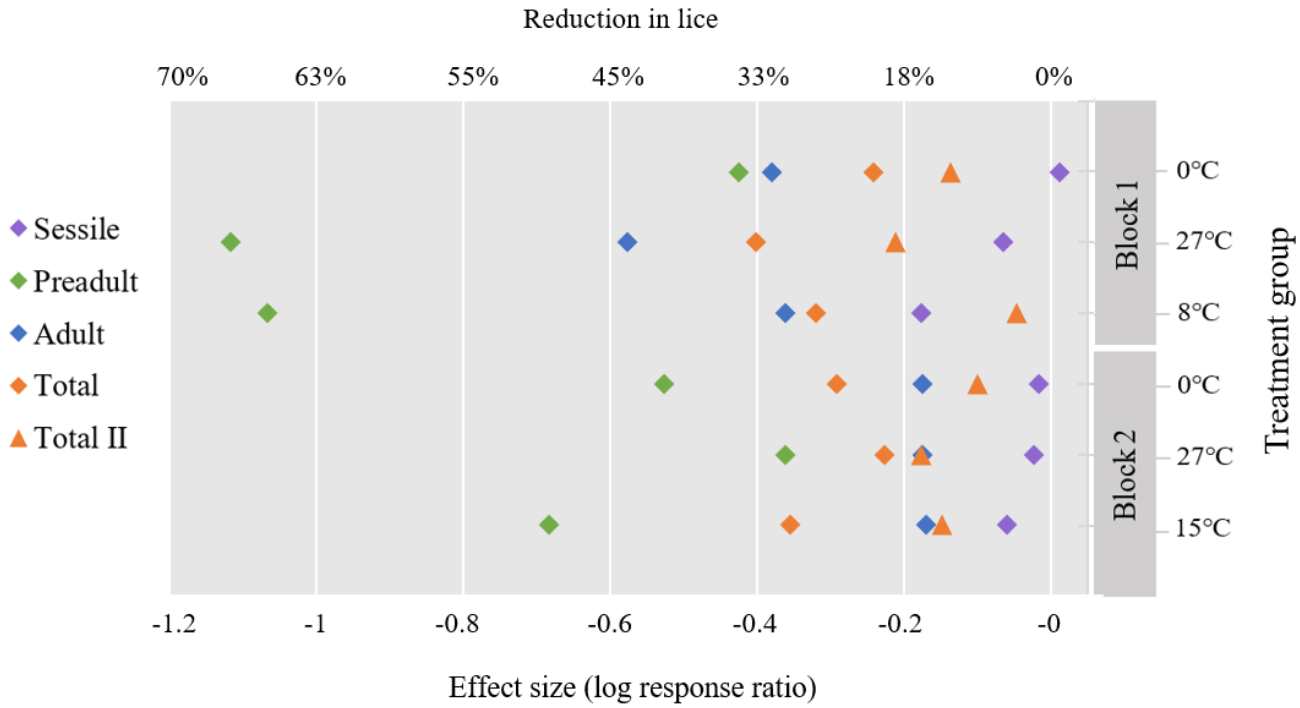


Figure 3.1.1: *Comparison of delousing efficiency with waterfall on fish kept at two holding temperatures (block 1:8°C or block 2: 15°C) and with three treatment temperatures (0, 27 or 8/15°C). Sessile, preadult and adult shows efficiencies right after treatment, total shows both right after treatment and at the end of the trial (Total II). Total = mean (sessile + preadult + adult). Effect sizes shows relative delousing efficiency between treatments and reference groups, presented as a log response ratio: RR = ln(T/R), where T is the treatment groups and R is the reference (procedural control) groups. Negative effect sizes indicate delousing efficiencies.*

Before vs. after treatment

Before treatment there was no significant difference in preadult lice ($W \geq 12806$, $p \geq 0.352$) or adult lice ($W \geq 4480$, $p \geq 0.269$) between any of the groups in block 1, or in sessile lice ($W \geq 3600$, $p \geq 0.171$), preadult lice ($W \geq 3522$, $p \geq 0.209$) or adult lice ($W \geq 3095$, $p \geq 0.817$) between any of the groups in block 2.

There was no difference in the level of sessile lice for any of the groups after treatment compared to before in block 1 (Figure 3.1.2). For block 2, an increase in sessile lice for all groups after treatment was evident (Table 3.1.1, Figure 3.1.2). Though the increase was highest in the untreated group compared to the treated (85 % on average) in block 2, the difference was not significant (115 % vs. 85 %, $W = 10650$, $p = 0.284$).

For preadult lice, all groups in both blocks had a decrease in lice levels after treatment (Table 3.1.1, Figure 3.1.2). Although the untreated group in block 1 had a lower decrease than the treated (84 % on average), the difference was not significant (62 % vs. 84 %, W = 12441, p = 0.658). For block 2, the decrease was significantly lower for the untreated group compared to the average decrease of the treated groups (44 % vs. 65 %, W = 12640, p < 0.001).

For adult lice, all groups in block 1 except the untreated had a reduction in adult lice (Table 3.1.1, Figure 3.1.2). The difference in reduction between the untreated vs. treated groups (35 % on average) in block 1 was significant (0 % vs. 35 %, W = 16928, p < 0.001). Block 2 had an increase in adult lice for all groups (Table 3.1.1, Figure 3.1.2). Although the increase was highest in the untreated group compared to the treated groups (92 % on average), the difference was not significant (119 % vs. 92 %, Wilcox = 10572, p = 0.357).

For total lice levels, all treated group in both blocks had a significant reduction (Table 3.1.1, Figure 3.1.2). The average reduction of the treated groups was significantly higher than the untreated groups for both block 1 (W = 7303, p < 0.001) and block 2 (W = 6876, p < 0.001).

Additionally, when comparing the reduction in lice levels between fish treated with warm or cold water (0/27°C) compared to the procedural control (8/15°C), no significant difference was found right after treatment for most treatments in either of the blocks (Appendix 5).

Table 3.1.1: *Differences in lice levels (n = sum of lice) right after treatment compared to before treatment for fish kept at two holding temperatures, block 1: 8°C and block 2: 15°C. Total = sum (sessile + preadult + adult). Negative treatment efficiency equals a decrease in lice levels. Significance levels are p<0.05 (*), p<0.01 (**) and p<0.001(***).*

Block	Lice stage	Treatment temp (°C)	Treatment efficiency (%)	n lice before treatment	n lice after treatment	t-value	p-value
1	Sessile	0	+18	163	193	1.688	0.093
		27	+4	166	195	1.049	0.295
		8	0	172	172	-0.099	0.921
		Untreated	+19	167	198	1.496	0.136
	Preadult	0	-75	67	17	-4.987	< 0.001***
		27	-87	67	9	-5.541	< 0.001***
		8	-89	70	8	-5.397	< 0.001***
		Untreated	-62	69	26	-4.228	< 0.001***

	Adult	0	-31	399	275	-4.654	< 0.001***
		27	-42	410	237	-5.933	< 0.001***
		8	-33	437	294	-4.600	< 0.001***
		Untreated	0	401	401	-0.015	0.988
	Total	0	-23	629	485	-4.118	< 0.001***
		27	-31	643	441	-5.733	< 0.001***
		8	-30	679	474	-4.814	< 0.001***
		Untreated	-2	637	625	-0.305	0.760
2	Sessile	0	+70	54	92	3.624	< 0.001***
		27	+113	47	100	4.081	< 0.001***
		15	+73	52	90	3.492	< 0.001***
		Untreated	+115	46	99	5.360	< 0.001***
	Preadult	0	-66	432	149	-9.858	< 0.001***
		27	-56	434	189	-10.340	< 0.001***
		15	-73	507	135	-12.020	< 0.001***
		Untreated	-44	476	264	-7.374	< 0.001***
	Adult	0	+78	81	144	4.083	< 0.001***
		27	+91	86	164	4.297	< 0.001***
		15	+106	81	167	5.130	< 0.001***
		Untreated	+119	88	193	5.476	< 0.001***
	Total	0	-32	567	385	-4.715	< 0.001***
		27	-20	567	453	-4.343	< 0.001***
		15	-39	640	392	-6.049	< 0.001***
		Untreated	-9	610	556	-1.233	0.219

Before vs. end of trial - two weeks after treatment

The trends of development over time displayed the shifting from sessile to preadult stages in block 1 and more towards adults in block 2. The data analysis of the treatment results two weeks after focus on the total number of lice, as shown at the bottom of Figure 3.1.2. The difference in lice is calculated by comparing the total amount of lice 2-4 days pre-treatment with the total amount of lice two weeks after treatment.

Before treatment, there was no significant difference in the total amount of lice between any of the groups in block 1 ($W \geq 4466$, $p \geq 0.516$) or block 2 ($W \geq 3666$, $p \geq 0.313$). All the groups in both blocks had a significant decrease in the total amount of lice at the end of the trial (Table 3.1.2, Figure 3.1.2). The average decrease in lice for the treated groups in block 1 was >2x higher compared to the untreated group (24 % vs. 11 %, $W = 10195$, $p = 0.025$). In block 2, the difference in the reduction of lice between the untreated group and the treatment group with the highest efficiency (15°C) was significant (27 % vs. 42 %, $W = 2618$, $p = 0.014$).

Table 3.1.2: *Differences in total lice levels (n = sum of lice) at the end of the trial compared to before treatment for fish kept at two holding temperatures, block 1: 8°C and block 2: 15°C. Negative treatment efficiency equals a decrease in lice levels. Significance levels are p<0.05 (*), p<0.01 (**) and p<0.001(***).*

Block	Treatment temp (°C)	Treatment efficiency (%)	n lice prior to treatment	n lice at the end of trial	t-value	p-value
1	0	-26	669	492	-4.258	< 0.001***
	27	-30	671	472	-4.736	< 0.001***
	8	-20	692	557	-3.149	0.002**
	Untreated	-11	643	571	-2.003	0.047*
2	0	-37	674	425	-4.430	< 0.001***
	27	-40	686	413	-5.346	< 0.001***
	15	-42	708	414	-4.879	< 0.001***
	Untreated	-27	697	510	-3.628	0.001**

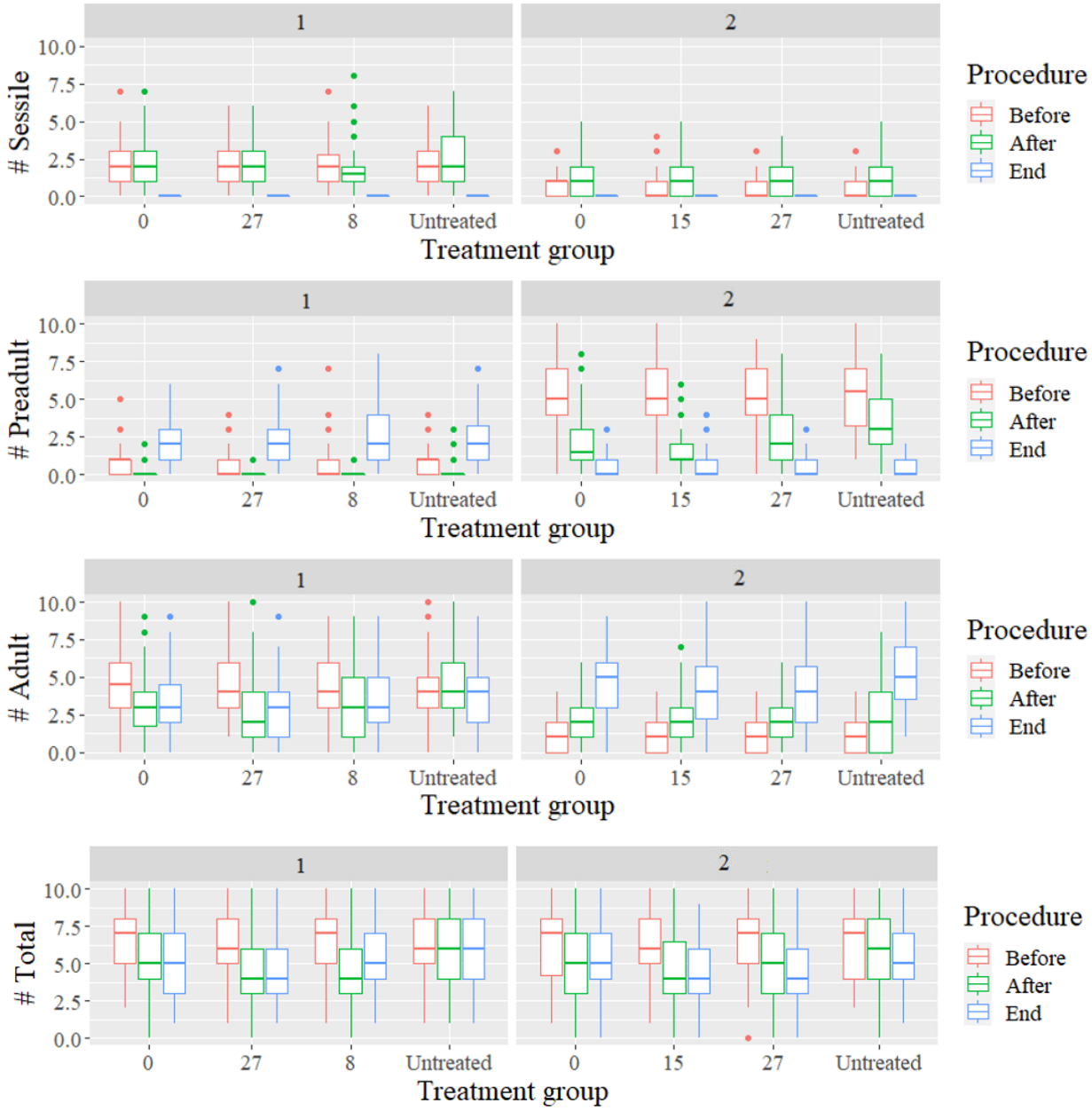


***Figure 3.1.2**: Lice levels before, right after treatment and at the end of the trial of fish kept at two holding temperatures (1 = block 1: 8°C, 2 = block 2: 15°C) and with three treatment temperatures (8/15, 0 or 27°C). In the boxplot, the upper line represents the 75 % quantile, middle line: median, 50 % quantile, and lower line: 25 % quantile. Outliers are represented by the coloured dots.*

3.2 Mortality and size effect

Mortality

Mortality throughout the trial was most prominent in block 2 (15℃). A total of 17 fish died in the tank after treatment on the same day (Table 3.2.1). Two fish died between the treatment day and the final evaluation two weeks post treatment, both from the 0℃ group in block 2. Six fish jumped out of the waterfall during treatment and were consequently euthanized. These fish were excluded from the mortality calculations.

For block 1 there was no mortality, neither right after treatment nor until the end of the trial. Two fish jumped out of the waterfall and were euthanized.

Table 3.2.1: *Number of fish dead after treatment on the same day in block 2 (15℃), divided into treatment groups. Not identified = pit tag missing.*

Treatment group	0℃	15℃	27℃	Untreated	Not identified
n fish dead	3	4	6	2	2

Size effect

When investigating size effect, a negative correlation between delousing efficiency and fish size was found (Spearman, $\rho = -0.115$, $p = 0.009$), as shown in Figure 3.2.1. For the analysis, fish size was defined by weight (g) and the delousing efficiency was equal to the reduction in the total number of lice from before treatment to the end of the trial for the treated groups. When analysing the size effect of the individual treatments from block 1 and 2 separately, a significant correlation was found for the 27℃ treatment in block 2 (Spearman, $\rho = -0.345$, $p = 0.002$)

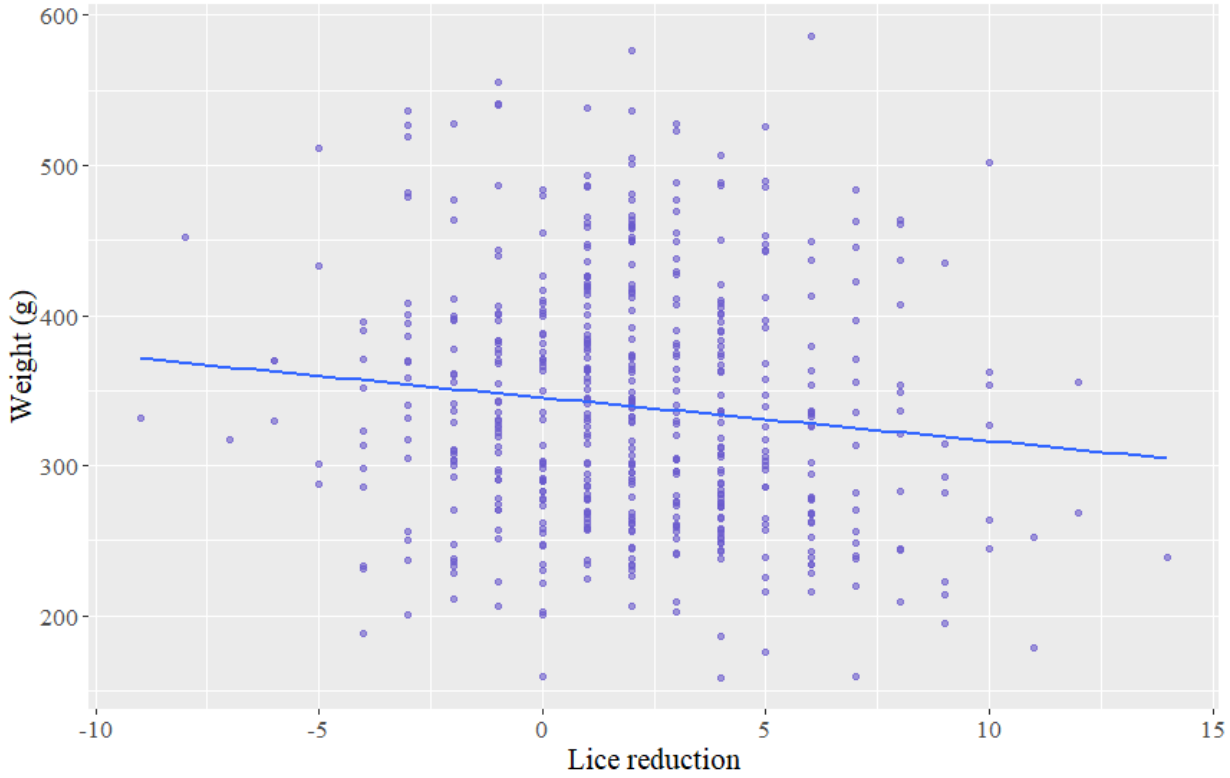


***Figure 3.2.1**: Correlation between delousing efficiency (lice reduction) and weight (g) of fish kept at two holding temperatures (block 1: 8°C, block 2: 15°C) and treated with waterfall at three temperatures (0, 27 or 8/15°C). Lice reduction = lice at the end of the trial (two weeks post treatment) – lice at pit tagging (before treatment). Regression line: y = 345.402 -2.892x. Spearman's correlation coefficient: ρ = -0.115, p-value = 0.009.*

3.3 Condition and welfare

Welfare before treatment

Prior to treatment, there was no significant difference in welfare score between any of the groups in either of the blocks for the welfare parameters: snout damage, eye bleeding, skin bleeding, scale loss, fin bleeding or fin erosion (Figure 3.3.1, Appendix 7). Also, there was no difference in scores between groups for fin splitting in block 1, for block 2 the difference was not significant after Bonferroni correction (Appendix 7).

Welfare after treatment

The scores for snout damage were higher after treatment (Figure 3.3.1), but only for block 1 with the 8 ℃ group having significantly higher scores than the untreated group ($W = 4819$, $p = 0.026$).

For eye bleeding, scores were higher after treatment for all treated groups in block 1 and all groups in block 2 (Table 3.3.1, Figure 3.3.1). The untreated group had lower scores than the treated groups in both block 1 ($W = 13793$, $p = 0.027$) and block 2 ($W = 11763$, $p < 0.001$).

An increase in welfare score is noticeable for skin bleeding in the treated groups in block 1 and all groups in block 2 (Table 3.3.1, Figure 3.3.1). Post treatment in block 1, the treated groups had higher scores than the untreated group ($W = 14517$, $p < 0.001$) and the 8℃ group had significantly lower scores than the 27℃ group ($W = 3547$, $p = 0.026$). In block 2, there were no difference between any of the groups after treatment.

For scale loss there was an increase in scores for all treated groups in block 2 (Table 3.3.1, Figure 3.3.1). The treated groups had higher scores after treatment than the untreated ones for both block 1 ($W = 13322$, $p = 0.025$) and block 2 ($W = 12024$, $p < 0.001$).

The scores for fin bleeding did not change much after treatment, only the 27℃ group in block 2 had a significant increase (Table 3.3.1, Figure 3.3.1). Thus, a significant difference between groups can only be found in block 2, where the treated groups had higher scores than the untreated ($W = 12195$, $p < 0.001$).

An increase in scores for fin splitting was only evident in block 2 (all groups) after treatment (Table 3.3.1, Figure 3.3.1), with no significant difference between any of the groups ($W \leq 11192$, $p \leq 0.061$).

Only the 27℃ group in block 1 had a significant increase in welfare score in terms of fin erosion after treatment (Table 3.3.1, Figure 3.3.1), with no significant difference between any of the groups ($W \leq 4443$, $p \leq 0.218$).

Additionally, no significant difference between fish treated with warm or cold water (0/27℃) compared to the procedural control (8/15℃) was found right after treatment for either of the blocks (Appendix 8).

Table 3.3.1: *Significant increase in welfare scores (using the FISHWELL-scoring schema) right after treatment compared to before treatment, assessed by a Wilcoxon rank sum test. Fish were kept at two holding temperatures (block 1: 8℃ and block 2: 15℃) and had three treatment temperatures 0, 27 or 8/15℃.*

Welfare parameter	Block	Relevant groups	Wilcox-value	p-value
Snout damage	1	All	≤ 1945	< 0.001
	2	-	-	-
Eye bleeding	1	Treated	≤ 27195	≤ 0.017
	2	All	≤ 2488	< 0.001
Skin bleeding	1	Treated	≤ 3595	≤ 0.013
	2	All	≤ 1897	< 0.001
Scale loss	1	-	-	-
	2	Treated	1852	< 0.001
Fin bleeding	1	-	-	-
	2	27℃	3868	0.011
Fin splitting	1	-	-	-
	2	All	≤ 2374	≤ 0.024
Fin erosion	1	27℃	5009	0.018
	2	-	-	-

Welfare end of trial

Compared to before treatment, most groups had higher scores at the end of the trial (Table 3.3.2, Figure 3.3.1). For snout damage, all groups in both blocks had higher scores while for eye bleeding, only the groups in block 2 had higher scores (Table 3.3.2). For skin bleeding, all groups in block 2 had higher welfare scores, but only the 0℃ and 8℃ from block 1 (Table 3.3.2). For scale loss, there was no difference in scores for any of the groups in either of the blocks (Table 3.3.2). For fin bleeding, only the groups in block 2 had higher scores and for fin splitting, only the 15℃ from block 2 had higher scores (Table 3.3.2). For fin erosion, the 27℃ group from block 1 had lower scores while the untreated group had higher scores (Table 3.3.2). The 15℃ group in block 2 also had lower scores in terms of fin erosion at the end of the trial (Table 3.3.2).

Table 3.3.2: *Significant increase in welfare scores (using the FISHWELL-scoring schema) at the end of the trial compared to before treatment, assessed by a Wilcoxon rank sum test. Fish were kept at two holding temperatures (block 1: 8℃ and block 2: 15℃) and had three treatment temperatures 0, 27 or 8/15℃.*

Welfare parameter	Block	Relevant groups	Wilcox-value	p-value
Snout damage	1	All	≤ 6813	< 0.001
	2	All	≤ 3965	≤ 0.001
Eye bleeding	1	-	-	-
	2	All	≤ 3822	≤ 0.017
Skin bleeding	1	0℃	4541	0.002
		8℃	4836	0.018
	2	All	≤ 3805	< 0.001
Scale loss	1	-	-	-
	2	-	-	-
Fin bleeding	1	-	-	-
	2	All	≤ 3987	≤ 0.003
Fin splitting	1	-	-	-
	2	15℃	3761	0.038
Fin erosion	1	Untreated	3277	≤ 0.029
		27℃!	3097!	0.001!
	2	15℃!	2652!	0.044!

! = A significant decrease was found rather than an increase.

Compared to right after treatment, a majority of the groups had lower scores at the end of the trial (Table 3.3.3, Figure 3.3.1). For snout damage, the groups in block 2 had higher scores (Table 3.3.3). For eye bleeding, all the treated groups in block 1 had lower scores, as well as the 0℃ and 15℃ groups in block 2 (Table 3.3.3). For skin bleeding, only the 27℃ group from block 1 had lower scores as well as all treated groups in block 2 (Table 3.3.3). For scale loss, all treated groups in block 2 had lower scores (Table 3.3.3). For fin bleeding, the untreated group from block 2 had higher scores, rather than lower (Table 3.3.3) For fin splitting, the untreated group from block 1 had lower scores, as well as the treated groups from block 2 (Table 3.3.3). For fin erosion, the 15℃ and 27℃ groups from block 2 had lower scores (Table 3.3.3).

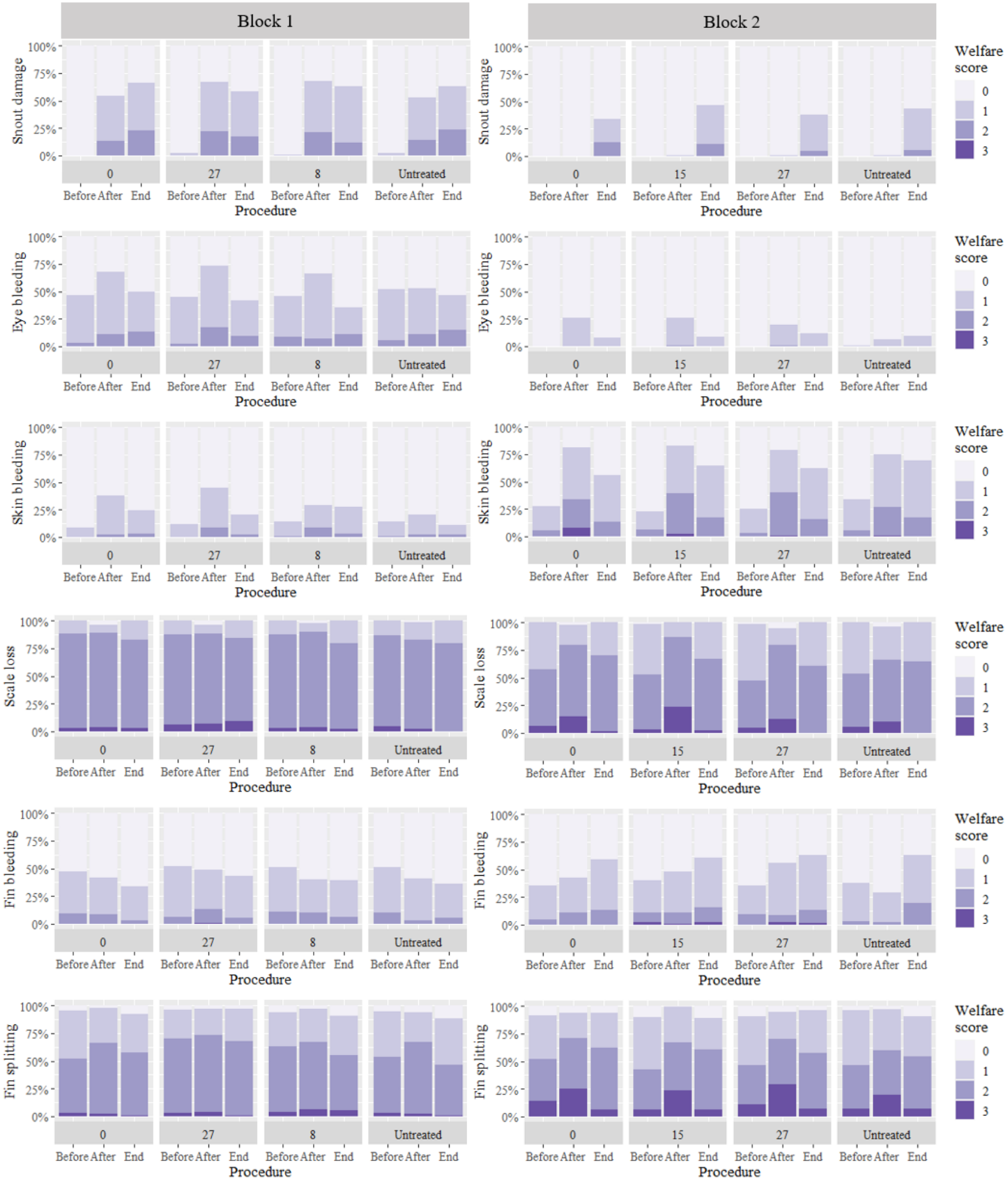
Table 3.3.3: *Significant decrease in welfare scores (using the FISHWELL-scoring schema) at the end of the trial compared to right after treatment, assessed by a Wilcoxon rank sum test. Fish were kept at two holding temperatures (block 1: 8°C and block 2: 15°C) and had three treatment temperatures 0, 27 or 8/15°C.*

Welfare parameter	Block	Relevant groups	Wilcox-value	p-value
Snout damage	1	-	-	-
	2	All!	≤ 3966!	< 0.001!
Eye bleeding	1	Treated	≤ 3154	≤ 0.020
	2	0°C	2387	0.002
		15°C	2636	0.004
Skin bleeding	1	27°C	3130	< 0.001
	2	Treated	≤ 2302	< 0.001
Scale loss	1	-	-	-
	2	Treated	≤ 2357	≤ 0.008
Fin bleeding	1	-	-	-
	2	Untreated!	4897!	< 0.001!
Fin splitting	1	Untreated	3057	0.003
	2	Treated	≤ 2424	≤ 0.033
Fin erosion	1	-	-	-
	2	15°C	2338	0.002
		27°C	2415	0.009

! = A significant increase was found rather than a decrease.

Comparing the scores between treated and untreated groups at the end of the trial, no significant difference was found for any of the welfare parameters: snout damage, eye bleeding, scale loss, fin bleeding or fin erosion in either of the blocks (Appendix 9a). Only for skin bleeding, the untreated group had lower scores than the 0°C ($W = 4404$, $p = 0.030$) and 8°C ($W = 4780$, $p = 0.005$) group in block 1. For fin splitting, the 27°C-group had higher welfare scores than the untreated group in block 1 ($W = 5029$, $p = 0.003$).

Additionally, no significant difference between fish treated with warm or cold water (0/27°C) compared to the procedural control treatments (8/15°C) was found at the end of the trial for either of the blocks (Appendix 9b).


Block 1
Block 2
Snout damage
Eye bleeding
Skin bleeding
Scale loss
Fin bleeding
Fin splitting
100%
75%
50%
25%
0%
0
27
8
15
Untreated
Before After End
Procedure
Welfare score
0
1
2
3

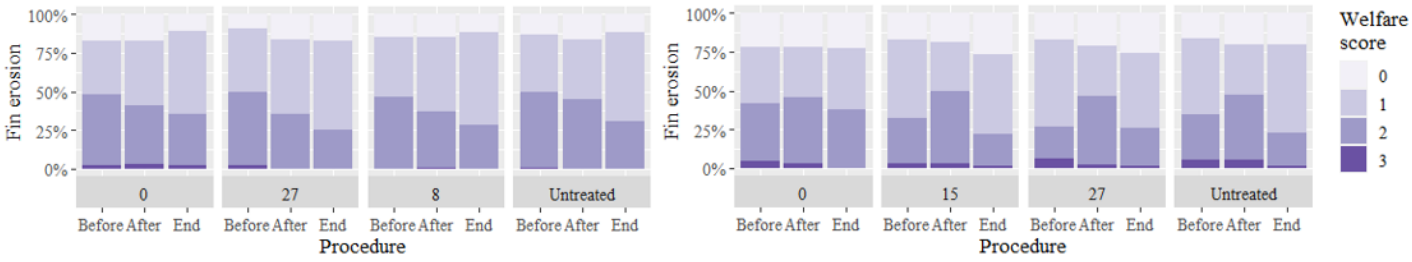


Figure 3.3.1*: Distribution by percentage of welfare scores before, right after treatment and at the end of trial based on selected welfare parameters using the FISHWELL-scoring schema. Fish were kept at two holding temperatures (block 1: 8°C or block 2: 15°C) and had three treatment temperatures (0, 27 or 8/15°C).*

4. Discussion

4.1 Discussion of materials and methods

Experimental design

Optimally for the experimental setup there would have been several tanks for each treatment group. Unfortunately, due to a limited number of tanks available it was not possible to separate the treatment/control groups in different tanks. Hence, a common garden design with pit tagged individuals was considered the best solution. Pit tagging the fish allowed for a follow up of individual lice levels and welfare, reducing tank space by keeping all the fish from each block in the same holding tank, while simultaneously reducing the animals needed for the trial. Reducing the number of experimental animals is also in accordance with the 'refinement' element in the guidelines for use of animals in scientific research, which aims to minimise potential pain, suffering or distress.

Lice development

The development of the salmon lice was slightly different between the two blocks. Block 1 (8°C) had more developed lice than block 2 (15°C) by the time of all count samples. Thus, most of the sessile lice in block 2 were chalimus I at the 2-4 days pre-treatment pit tagging procedure, while most of the lice in block 1 had become chalimus II. Equivalent development shifts were seen at preadult and adult stages. Block 1 had more adults and less preadults at pit tagging than block 2, which had less adults and more preadults. The development of lice between stages at the different times of the procedures makes for a challenge regarding the estimation of delousing efficiency.

To mitigate this issue, the data was analysed in two different ways both per stage and in total, where delousing efficiency was calculated as described in section 2.7. The first method used the number of lice on untreated, procedural control groups as reference (Figure 3.1.1) for the results to be comparable to previous trials and to correct for the effect of the procedure itself. The second method used the number of lice 2-4 before treatment (at pit-tagging) as reference (Figure 3.1.2), comparing lice levels before treatment with both after treatment and end of trial.

Both methods have pros and cons. Using lice numbers from pit-tagging may be more inaccurate due to the development of the lice between the tagging and the treatment, but it allows for comparison of the change in lice levels between treated and untreated groups. Meaning it is possible to investigate how much of the delousing efficiency is caused by the waterfall treatment itself and how much is caused by other procedural factors such as crowding and handling. This method may also be more practical in a larger production where it is not possible to have a procedural control group. While if only using control groups as reference may be more accurate in terms of delousing efficiency calculations, one is very dependent that the variance between control- and treatment groups are minimal. As done in the present study, including both ways were therefore considered most optimal.

Technical sources of error

Technical sources of error include accuracy in water pressure during treatment and accuracy in lice counting. During the delousing procedure, the pressure in the waterfall was constantly monitored to be as close to 0.6 bar as possible. However, for some of the treatments the water pressure fell slightly below the intended pressure level, shown in Appendix 3. This might be caused by inconsistency of the pump itself, which was mostly a problem during the treatment of block 2. The clamps which secured the hose to the pump loosened slightly during the first day of treatment and were tightened for the second day, although it did not make too much of a difference.

The pressure was most unstable in the lowermost nozzle, showing 0.2 bar at the least (in 3/12 treatments). For the next waterfall procedure (block 1), the pressure was considerably more stable throughout both days of treatment, with only two treatments having nozzles showing a lower pressure of 0.4 - 0.5 bar. The pressure problems might have affected the delousing efficiency, but it is hard to estimate exactly to which extent. Considering the delousing efficiency, which were almost the same for treated groups in both blocks (Figure 3.1.1), it does not seem like the difference in pressure played a significant role.

The possible source of error in the accuracy of lice counting is regarding my own experience. To maximise consistency and minimize the source of error as much as possible, I counted all the lice in all the procedures throughout the trial (as well as did all the welfare scoring) myself. However, my experience in lice counting prior to the trial was limited, thus the counting accuracy naturally increased as the trial progressed. This probably explains the

increase is sessile lice after treatment seen in block 2, where many sessile chalimus I lice were observed at pit tagging. However, this possible source of error is not present when comparing the lice count of treated vs. untreated on the same day, where an expected decrease in sessile lice was evident (Figure 3.1.1). Inexperience in lice counting is probably also of less importance for the later preadult and adult life stages as these lice are significantly larger and easier to see, especially compared to chalimus I. However, more practice in lice counting prior to trial would be recommended for future studies.

Statistical analyses

In the statistical analysis, the Wilcoxon rank sum test was used to check for significant differences before vs. after/end, and between treated vs untreated groups. A weakness in using this type of non-parametric test is regarding multiple comparison, which involves controlling the Type 1 error rate. The more hypothesis tested, the higher the chance of at least one of the conclusions across the study being wrong. However, this test was used for the present study because it is not a classical multiple comparison situation due to the expectation of the results when looking at the bigger picture. To control the error rate, Bonferroni correction was used when a multiple comparison problem was suspected.

4.2 Effect of waterfall treatment on lice

When examining the effect of the waterfall treatment on lice levels, the two methods of analysis using different reference groups (described in section 2.7) are used in various parts of the discussion, to collectively investigate three main effects: overall delousing efficiency, main effects of the waterfall treatment itself and the effect of temperature in the treatment water.

4.2.1 Delousing efficiency

To estimate the general delousing efficiency of the waterfall treatment, both short- and long term, the analysis using lice levels on untreated, procedural control groups as reference was used (Figure 3.1.1). Because all the counts used for the analysis is from the same day, it is not affected by lice development and will thus give the most accurate estimation.

The results right after treatment suggest minimal to no delousing effect on sessile lice for any of the treatments in either of the blocks, with no significant difference between groups. The delousing effect on preadult lice seems to be the highest among the different stages, with an average of 56 % for block 1 and 40 % for block 2. The efficiency on adult lice is lower, with an average of 35 % for block 1 and 16 % for block 2. Hence, the waterfall treatment seems to have no overall effect on sessile lice and moderate effect on preadult/adult stages. How the results compare to the previous waterfall trials is discussed in detail in section 4.3.

Although the estimated delousing efficiencies is not sufficient for commercial use, it should be noted that the current trial is a small-scale experimental setup compared to commercial, mechanical delousing procedures. Large-scale delousing procedures include extra factors such as starving, crowding (for hours), pumping, and dewatering both prior to and after exposure to the treatment. These additional elements further increase the delousing effect. Thus, the delousing effect caused by the commercial treatment itself would be added to the treatment efficiency estimated in the present trial, which could be interesting for further prospects.

When investigating the long-term delousing efficiency of the waterfall treatment, the results are mainly focused on the total number of lice rather than the individual stages. The delousing efficiency at the end of the trial was lower compared to right after trial (Figure 3.1.1), going from 27 → 19 % for block 1 and 25 → 16 % for block 2. Suggesting that there was no delayed effect of the treatments, which would have been shown as a higher reduction in lice at the end of trial compared to right after treatment. The slight increase in lice by the second counting is most likely due to the fact that the original lice numbers were higher than the first count showed and that the countability of larger, mobile lice was higher. Similar increases in lice numbers has also been found with repeated counts in previous experiments (Torgersen 2016; Samsing et al. 2015).

4.2.2 Main effect of waterfall

When investigating the effect of the waterfall treatment itself, analysis using lice levels before treatment (at pit tagging) is used (Figure 3.1.2), to be able to compare the reduction in lice on treated groups vs. untreated groups. This comparison allows us to estimate how much of the delousing effect is due to the waterfall treatment itself, and how much is caused by other procedural factors such as crowding and handling. Thus, the difference in lice levels between treated and untreated groups, indicates the effect of the waterfall alone.

The results suggest that the waterfall treatment itself causes most of the delousing efficiency. For preadult lice, the treated groups had significantly higher delousing efficiency than the untreated in block 2, suggesting that the treatment accounts for ~30 % of the delousing efficiency. For adult lice, block 1 had a significant difference between treated and untreated groups, suggesting that ~100 % of the reduction in lice is caused by the waterfall treatment. The high percentage is caused by 0 % reduction in lice for the untreated group, resulting in the waterfall treatment effect accounting for the total efficiency. Furthermore, when looking at the difference in total amount of lice, a significant difference between treated and untreated groups were evident for both blocks. Indicating that the treatment made up 93 % of the total lice reduction in block 1 and 70 % in block 2. The latter estimations may be the more accurate, considering that when looking at the total amount of lice rather than the individual stages, the issue with lice development is mitigated. Thus, the results suggest that the waterfall treatment itself accounts for >70 % of the total delousing efficiency.

4.2.3 Effect of treatment water temperature (ΔT)

To investigate whether the temperature in the treatment water influenced delousing efficiency, lice levels on groups treated with warm- and cold-water (0/27°C) was compared with procedural control groups treated with the same temperature as the fish was acclimatized to (8/15°C for block 1/2 respectively). Both methods of analysing the data was used (Figure 3.1.1, Figure 3.1.2), due to no issues with lice development when comparing groups with each other.

The combined result from each analysis indicates that there is no effect of ΔT of the treatment water. When using lice levels on untreated groups as reference (Figure 3.1.1), the results of which treatment groups had higher delousing efficiency varied considerably, showing no common pattern (statistical tests in Appendix 4). Furthermore, when using lice level before treatment as reference (Figure 3.1.2), no overall difference was found between groups treated with warm/cold water compared to procedural control groups (Appendix 5), suggesting no visible effect of ΔT. Thus, the results indicates that the delousing efficiency is mostly caused by the mechanical treatment rather than the temperature of the treatment water itself.

The fact that the treatment water temperature had no impact on delousing efficiency may be the cause of short exposure time. In the current trial, the exposure time of the fish to the

treatment water were considerably shorter than for previous trials where the warm/cold water treatment (bath) were followed by, or prior to the waterfall treatment itself. For the present trial, the exposure time was only 3-5 seconds compared to 30 seconds (thermal bath, 32.5°C) or 3 hours (freshwater bath). Hence, implementing the warm/cold water in the waterfall treatment did not give the water temperature enough time to affect the lice, and consequently did not give the desired delousing effect.

4.3 Delousing effect compared to previous waterfall trials

In comparing the treatments to previous waterfall trials, only data from efficiencies calculated from untreated, procedural control groups was used. Delousing efficiency on both sessile and mobile lice were generally lower (Figure 4.3.1). It should be noted that Torgersen's (2016, 2017) treatments combined waterfall with pre-bath treatment (consisting of either fresh water or warm water). It is more relevant comparing the results of the current trial with delousing efficiencies from previous trials excluding the bath treatments, considering the difference in exposure time the fish has to the water between the trials as discussed in 4.2.3.

4.3.1 Current trial compared to 1st waterfall trail

Comparing the first waterfall trial (Torgersen 2016) with the current trial, there seems to be slight differences in delousing efficiencies (Figure 4.3.1). From block 1 (8°C) in the current trial, the delousing effect on sessile lice was 7 % on average for the treated groups, lower than the 31 % efficiency found in the previous trial with waterfall treatment alone (Figure 4.3.1). The average delousing effect on mobile lice in the present trial was 46 % for treated groups, which is similar to Torgersen's result of 42 % on mobile lice after waterfall treatment (Figure 4.3.1). The first trial used freshwater with raw water temperature (9°C) as treatment water, different to the salt water used in the present trial. Additionally, the waterfall used in the first trial was a prototype which was later modified to control pressure in the nozzles more accurately. Thus, although the first waterfall trial had a control group with only the waterfall treatment, direct comparison to the current trial is still problematic due to the modifying of the prototype, which could be the cause of the difference in delousing efficiency (mainly sessile) between the trials.

4.3.2 Current trial compared to 2nd waterfall trial

Although the same prototype and treatment intensity (0.6 bar) was used for both the second waterfall trial (Torgersen 2017) and the current trial, comparing the delousing efficiency is more complicated due to the lack of a control group using only waterfall as treatment from Torgersen's trial. Also, only block 1 from the current trial was used for comparison due to the similar holding temperatures (Torgersen: 8.5 – 9°C vs. current trial, block 1: 8°C).

The delousing efficiencies in the second waterfall trial (Figure 4.3.1) indicates that the effect of the bath treatments (freshwater or thermo) by itself represents the greater part of the lice reduction in the combined bath- and waterfall treatments. Hence, when subtracting the efficiency of the freshwater/thermo baths Torgersen's results indicates a delousing effect of around 5-12 % for sessile and 7-33 % for mobile lice, suggesting that the effect of the waterfall treatment alone was not very large. Thus, compared to Torgersen's results, the average reduction of 7 % for sessile and 46 % for mobile lice in the present trial can seem promising (Figure 4.3.1).

Regardless, it is evident that the effect of warmer water (32.5°C) is significant compared to what was used in the current trial (27°C). However, warm water is thought to be painful for the fish (Nilsson et al. 2019) and temperatures above 28°C may not be allowed to use. A potential solution to this problem could be to use "painkillers" to maintain fish welfare while using higher treatment temperatures. A study by Folkedal et al. (2021) showed that anesthetized, small salmon post smolts had alleviated behavioural responses to thermal treatment with strong appetite within hours and negligible mortality.

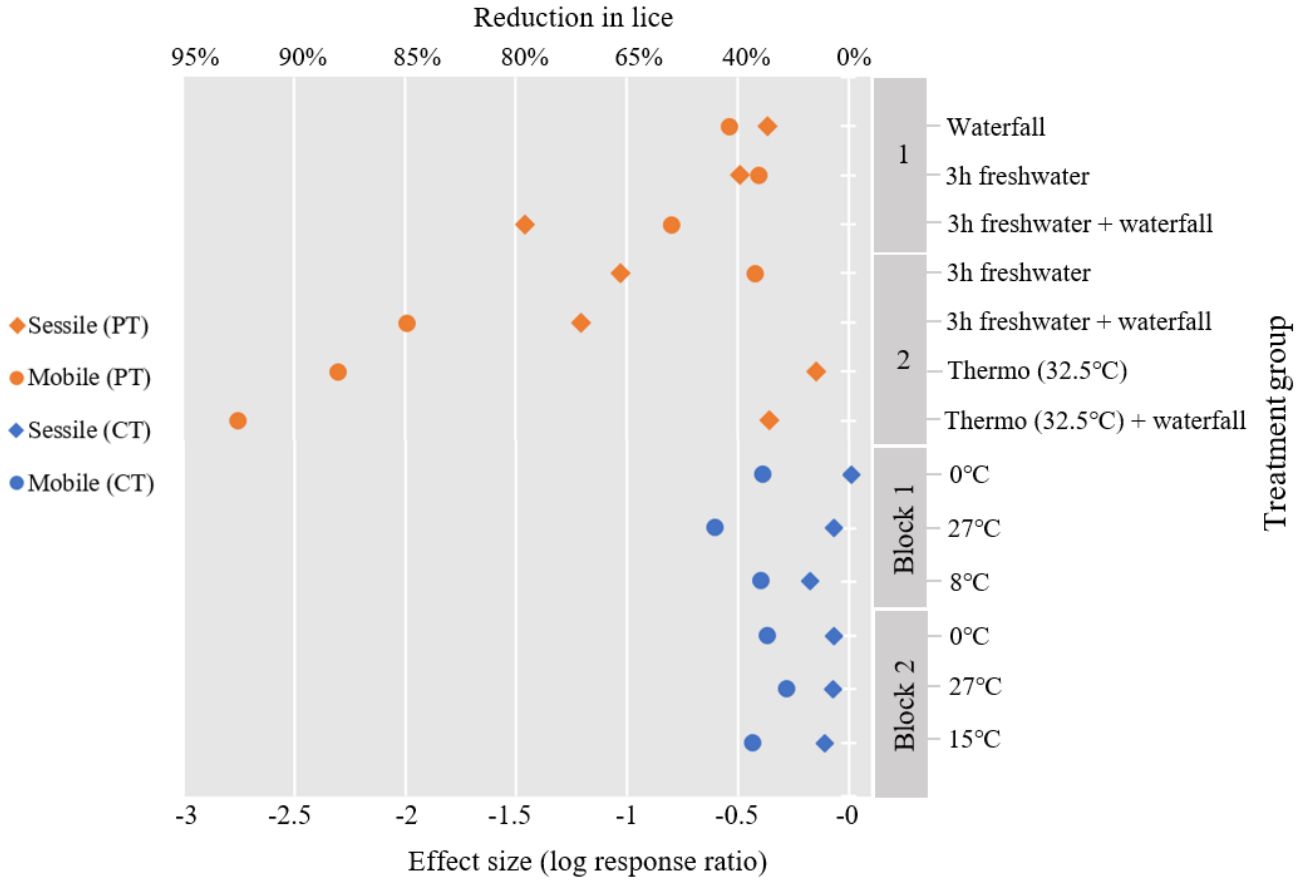


Figure 4.3.1: *Efficiency of delousing with waterfall from previous trials (PT, 1:Torgersen 2016 and 2:Torgersen 2017) compared to the current trial (CT). Current trial: fish kept at two holding temperatures (block 1:8°C or block 2: 15°C) and had three treatment temperatures (0, 27 or 8/15°C). Effect sizes shows relative delousing efficiency between treatments and reference groups, presented as a log response ratio: RR = ln(T/R), where T is the treatment groups and R is the reference groups. Negative effect sizes indicate delousing efficiencies.*

4.4 Mortality and size effect

Mortality throughout the trial

Mortality was only noticeable at high temperature, where 5 % died between the waterfall procedure and the final evaluation two weeks post treatment. Which is in accordance with the results from the previous waterfall trials, with a mortality of 3 % over a period of 15 days (Torgersen 2016) and had 8 % over 12 days (Torgersen 2017).

All fish in both blocks came from the same tank prior to being used in the current trial and underwent the same procedures throughout, the only difference between the blocks being the two different tanks of holding temperature. Higher mortality post treatment for fish held at

higher temperatures, 15°C vs. 8°C, has also been reported in earlier studies with mechanical delousing. According to Overton et al. 2018, mortality generally increased with temperature after both thermal and mechanical treatment where salmon at 13-16°C had higher mortality post treatment than fish at 7-10°C.

Effect of fish size on delousing efficiency

When investigating size effect, only a very small negative correlation ($\rho = 0.009$) between delousing efficiency and fish size was found (Figure 3.2.1), meaning that smaller fish had slightly higher reduction in the total amount of lice than bigger fish. For individual treatment groups, a significant negative correlation was only found for the 27°C treatment in block 2. If the reason was the temperature itself (i.e., caused by fish twisting and turning as a reaction to the warmer water (Nilsson et al. 2019)), a correspondingly correlation for block 1 should also have been evident. The significant correlation for this treatment group could be a coincidence, caused by a multiple comparison problem. However, after Bonferroni correction, the p-value was still significant ($p = 0.01$). A possible explanation could be that this group randomly had a lower mean weight and higher mean reduction in lice compared to all the other groups. Thus, follow-up experiments are needed to clarify whether the effect is real, and what may have caused it.

4.5 Condition and welfare

The welfare parameters not presented in the results (Appendix 6); cataract, protruding eyes, operculum damage and acute/bacterial wounds were generally low throughout the trial with WI-scores ≤ 1 overall for all groups in both blocks.

4.5.1 Short term effect of waterfall treatment on welfare

Welfare scores for some parameters were higher right after treatment compared to before treatment, but with differences between the blocks (Figure 3.2.2). For block 1, the scores for snout damage (all groups), eye bleeding (treated groups), skin bleeding (treated groups) and fin erosion (27°C group) were higher right after treatment. However, when comparing the treated vs. the untreated groups, a significant difference was only evident for eye bleeding and skin bleeding, which indicates that these welfare parameters were the ones affected by the

waterfall treatment itself. Eye bleeding had around 20 % increase in score 1 and 5 % increase in score 2, while skin bleeding had approximately 25 % increase in score 1 and 5 % in score 2 (Figure 3.2.2). Due to no significant difference between treated and untreated groups for snout damage right after treatment compared to before, the elevated welfare scores may have been caused by other elements of the procedure, such as handling and crowding.

For block 2, the scores for eye bleeding, skin bleeding and fin splitting were higher right after treatment compared to before treatment for all groups, in addition to the treated groups for scale loss and the 27°C group for fin bleeding. The comparison of welfare scores of treated vs. untreated groups right after treatment shows a significant difference in scores for eye bleeding, scale loss and fin bleeding, indicating a negative effect of the waterfall treatment on these parameters. Eye bleeding had an increase of ~20 % in score 1, scale loss an increase of ~10 % in score 3 and fin bleeding and increase of ~10 % in score 1 (Figure 3.2.2). Thus, again suggesting that the increase in welfare scores for the remaining parameters; skin bleeding and fin splitting, was caused by other factors than the waterfall itself as mentioned for block 1.

Also, the significant increase in welfare score for fin splitting after treatment might be incorrect due to some of the groups already being significantly different before treatment (prior to Bonferroni correction of the p-values). Thus, it is important to be aware of a possible source of error. Either because this group had more fin splitting (e.g., because they were accidentally handled rougher than the rest) or due to variation by the person scoring (happened to be a little stricter for this group).

4.5.2 Long term effect of waterfall treatment on welfare

By the end of the trial the physical strain from the waterfall treatment should be less evident compared to right after treatment, which is reflected in most of the welfare parameters being unchanged or lower after 14 days (Figure 3.2.3). For block 1, eye bleeding (treated groups), skin bleeding (27°C) and fin splitting (untreated group) had lower scores two weeks after treatment compared to right after. However, as a general decrease in welfare scores was expected, there is no common pattern as to which groups improved or not over the different welfare parameters. Neither was there a significant difference in scores between treated and untreated groups for any welfare parameter in either of the blocks at the end of the trial. For block 2, eye bleeding (0/15°C), skin bleeding (treated groups), scale loss (treated groups), fin splitting (treated groups) and fin erosion (15/27°C) had lower scores at the end of the trail

compared to right after treatment. The opposite results can be found for snout damage. Right after treatment the scores were close to zero for all groups, which increased to ~30 % getting score 1-2 for all groups (Figure 3.2.3). The appearance of snout damage in block 2 two weeks post treatment is most likely caused by an increase in jumping and consequent collisions with the tank wall.

Comparing the welfare scores at the end of treatment to the scores before treatment, most parameters have increased scores (Figure 3.2.3). Indicating that there might be a long-term effect on welfare, as the degree of injury for most parameters did not go back to the status before treatment. Also, the increase in scores was considerably more noticeable in block 2 compared to block 1. When comparing the treated with the untreated groups at the end of the trial, there was no significant difference in welfare scores in either of the blocks. Which suggest that the increase was not caused by the waterfall treatment, but rather other procedural factors. This could be a combination of the repetitive crowding, handling and stress causing the scores to have increased over time.

4.5.3 Effect of treatment temperature on welfare

To further investigate the effect of the waterfall treatment on welfare, analysis comparing groups treated with warm or cold water (0°C or 27°C) with the procedural control treatment (8°C for block 1 and 15°C for block 2) was performed. Because the warm water used in the current trial did not exceed 28°C, no pain reaction was expected from the fish. Considering the exposure time for the fish to the warm or cold water is short-term (about four seconds), we also did not expect the temperature of the water to have time to cause damage to the fish.

No significant difference between fish treated with warm or cold water compared to the procedural control treatment was found, neither right after treatment nor at the end of the trial for either of the blocks (Appendix 8, 9b). Only one exception occurred in block 1, where the 27°C group had significantly higher scores than the 8°C group. Considering this was the only exception, it could be a false result caused by a multiple comparison problem. After Bonferroni correction, the p-value was no longer significant ($p = 0.12$). Taken into account, it would be reasonable to assume that there was no considerable negative effect of waterfall temperature on overall welfare.

4.5.4 Overall welfare

The overall scores were noticeably higher for scale loss, fin splitting and fin erosion, however it should be noted that the scores for these parameters were high from the very start of the trial and that the waterfall treatment itself caused no negative long-term effects as discussed in 2.5.2. Throughout the trial >50 % of the fish in both blocks had obvious scale loss, and ~20 % had severe scale loss after treatment in block 2, similar to the scores observed after mechanical delousing treatment from Gismervik et al. (2019) and which can be caused by crowding (Espmark et al. 2015). Scale loss is normal in commercial fish farming, seen by Madaro et al. (2021), where an average of 17 % of fish in commercial production had WI ≥ 2 from beginning of production to slaughter. Fin splitting and fin erosion had overall poor scores, where ~50 % in total had obvious damage for both blocks throughout the trial. Fin splitting had an additional ~25 % severe damage in block 2. Fin damage is often found in farmed salmonids (Stien et al. 2013) and an increase in fin condition after mechanical delousing treatment has also been seen in other studies (Gismervik et al. 2019; Bui et al. 2020). Although a large amount of the individuals had high scores, fin splitting appears to be a relative mild type of damage which leaves skeletal fin rays undamaged (Noble et al. 2012).

5. Conclusion and further studies

5.1 Delousing efficiency of warm and cold waterfall treatment

The waterfall treatment seemed to have no visible effect on sessile stages. However, the results suggest an overall delousing effect of 40-56 % on preadult lice and 16-35% on adult lice, but with no delayed effect two weeks post treatment.

Furthermore, when investigating how much of the delousing efficiency was caused by the waterfall itself compared to other procedural factors such as crowding and handling, the results suggest that the waterfall treatment alone accounts for >70 % of the total delousing efficiency.

No overall difference was found between groups treated with warm or cold water (0/27°C) compared to procedural control groups (8/15°C), showing no visible effect of ΔT. Thus, the treatment water temperature seemed to have no effect on lice levels, indicating that the delousing efficiency is mostly caused by the mechanical treatment.

5.2 Welfare effects of warm and cold waterfall treatment

The results suggest that the waterfall treatment had a somewhat negative short-term effect on some of the welfare parameters: eye bleeding, skin bleeding, scale loss, and fin bleeding. Which were expected, as the procedure is a mechanical delousing method which would most likely cause some physical strain on the fish. Furthermore, it was evident that the welfare improved during the time after treatment, as the majority of the scores at the end of the trial were either unchanged or lower than right after treatment.

Hence, although the welfare scores at the end of the trial were generally higher than before treatment, the waterfall treatment itself did not seem to be the cause of this increase, but rather the repetitive procedural factors such as crowding, handling and stress. Thus, the waterfall did not seem to have long-term consequences on the fish in terms of welfare.

The temperature in the waterfall treatment did not seem to have a significant overall effect on welfare, neither short- nor long term, as no significant differences were found between groups treated with warm or cold water compared to groups treated with the same temperature the fish were acclimatized to.

5.3 Further studies

The present trial was conducted to investigate if the efficiency of mechanical delousing with waterfall (low pressure flushing) can be increased using warm or cold water (27℃ or 0℃ respectively) in the treatment. The exposure time for the fish to the treatment water was short (only 3-5 seconds), most likely causing the results indicating that ΔT had no effect on the delousing efficiency. Thus, it would be interesting to further investigate if modifying the treatment to either include more extreme temperatures or increase the exposure time to the treatment water, would be a significant improvement of the waterfall treatment. Because warm water is thought to be painful for the fish (Nilsson et al. 2019), temperatures over 28 temperatures above 28℃ may not be allowed to use in the future. Hence, a potential prospect for further studies could also combine increased treatment temperature with the use of "painkillers" (Folkedal, Utskot, and Nilsson 2021), to maintain fish welfare. Furthermore, although the delousing efficiency found in the current trial is not sufficient for commercial use, it must be noted that a larger-scale procedure most likely would increase the effect considerably and should also be considered.

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Appendix

1. FISHWELL scoring schema

LUSETELLING OG VELFERDSSKÅR

CrowdMonitor

Forsøk	Lusefoss - pitmerking
Dato	
Bolk	
Bedøvelse	
Utført av	

Generelle skåringskriterier:

0	=	Intet å anmerke
1	=	Antydning / mistanke defekt
2	=	Tydelig skade / defekt
3	=	Betydelig skade, bør avlives

			Lus			Hode					Hud				Finner			Merknad
PIT	Lengde (cm)	Vekt (g)	Fastsittende	Preadult 1-2	Voksne	Snuteskade	Øyeblødning	Utstående øyne	Blakking	Gjellelokk-skade	Skjeltap	Hudblødning	Akutte sår	Bakteriesår	Blødming	Splitting	Erosjon	Beskrivelse av skade, finne med mest skade, andre skader, deformiteter, sykdomstegn etc.

2. Specifications for collected batches of egg strings

Overview of collected batches of egg strings. Tank nr. corresponds to Hall 4, in the Tank Environmental Lab (TEL) at Matre research station. Incubation was held at two different temperatures depending on when the copepodids were needed for infestation.

Date	Collected from tank nr.	Pairs of egg strings collected	Incubation temperature (°C)
03.06	15, 16	21	11
10.06	13	20	11!
12.06	14	20	15
19.06	15, 16	14	15
07.07	13, 14, 15, 16	20	15

! = Moved to 15 °C the following day.

3. Additional specifications for rounds of waterfall

Block 1: 8°C

The first day, two fish jumped out of the waterfall and was euthanized. Three jumped out on the second day. All tag-ID were scanned and noted. There were two fish that had lost their tags (unable to get their tag-ID). Fish were transferred to tank 6 after treatment and feeding was turned on 16:15 on the first day of the procedure. Approximately all the sessile lice that was counted was chalimus II. Additionally, some mature lice with egg strings were observed, something that was not seen before in the final outtake of block 2.

Treatment order for block 1:

Round	Treatment group (n = 25)			
1	0°C	Untreated	15°C	27°C
2	27°C	15°C	0°C	Untreated
3	Untreated	0°C	27°C	15°C
4	15°C	27°C	Untreated	0°C

Block 2: 15°C

Six fish jumped out of the waterfall in total, three each day, and were euthanized. All tag-IDs were scanned and noted. 17 fish died in the tank after treatment, eight on the first day and nine the second day. These were taken out and their tag-ID was scanned and noted. There were 18 fish that had lost their tags (unable to get their tag-ID). Fish were transferred to tank 4 after treatment and feeding was turned on 18.00 on the first day of the procedure. Approximately all the sessile lice that was counted had become chalimus II since the pit tagging procedure where most were closer to chalimus I. No mature female lice with egg strings were counted.

Treatment order for block 2:

Round	Treatment group (n = 25)			
1	27°C	Untreated	8°C	0°C
2	0°C	8°C	27°C	Untreated
3	Untreated	27°C	0°C	8°C
4	8°C	0°C	Untreated	27°C

Water pressure specification

Block	Round	Treatment group (°C)	n nozzles not at 0.6 bar	Measured pressure (bar)	Comments
1	1	27	3	0.5	
	4	0	1	0.4	Lowermost
2	2	27	2	0.4	
	2	0	1	0.2	Lowermost
	3	0	1	0.8	
	4	15	1	0.2	Lowermost
	4	27	1	0.5	Lowermost
	4	0	1	0.2	Lowermost

4. Lice levels: control vs. treatment

Comparison of delousing efficiency between groups right after treatment using lice levels on untreated, procedural control groups as reference. Significance levels are p<0.05 (*), p<0.01 (**) and p<0.001(***), assessed by two-proportion z-test.

Block	Lice stage	Compared treatments (°C)	p-value	Treatment with higher delousing efficiency (°C)
1	Sessile	0 – 27	!	!
		0 – 8	!	!
		27 – 8	!	!
	Preadult	0 – 27	0.015*	27
		0 – 8	0.015*	8
		27 – 8	1.000	-
	Adult	0 – 27	0.001**	27
		0 – 8	0.693	-
		27 – 8	< 0.001***	27
	Total	0 – 27	< 0.001***	27
		0 – 8	0.002**	8
		27 – 8	0.323	-
2	Sessile	0 – 27	1.000	-
		0 – 15	0.568	-
		27 – 15	0.767	-
	Preadult	0 – 27	0.001**	0
		0 – 15	0.197	-
		27 – 15	< 0.001***	15
	Adult	0 – 27	0.630	-
		0 – 15	0.267	-
		27 – 15	0.613	-
	Total	0 – 27	0.004**	0
		0 – 15	0.762	-
		27 – 15	0.001**	15

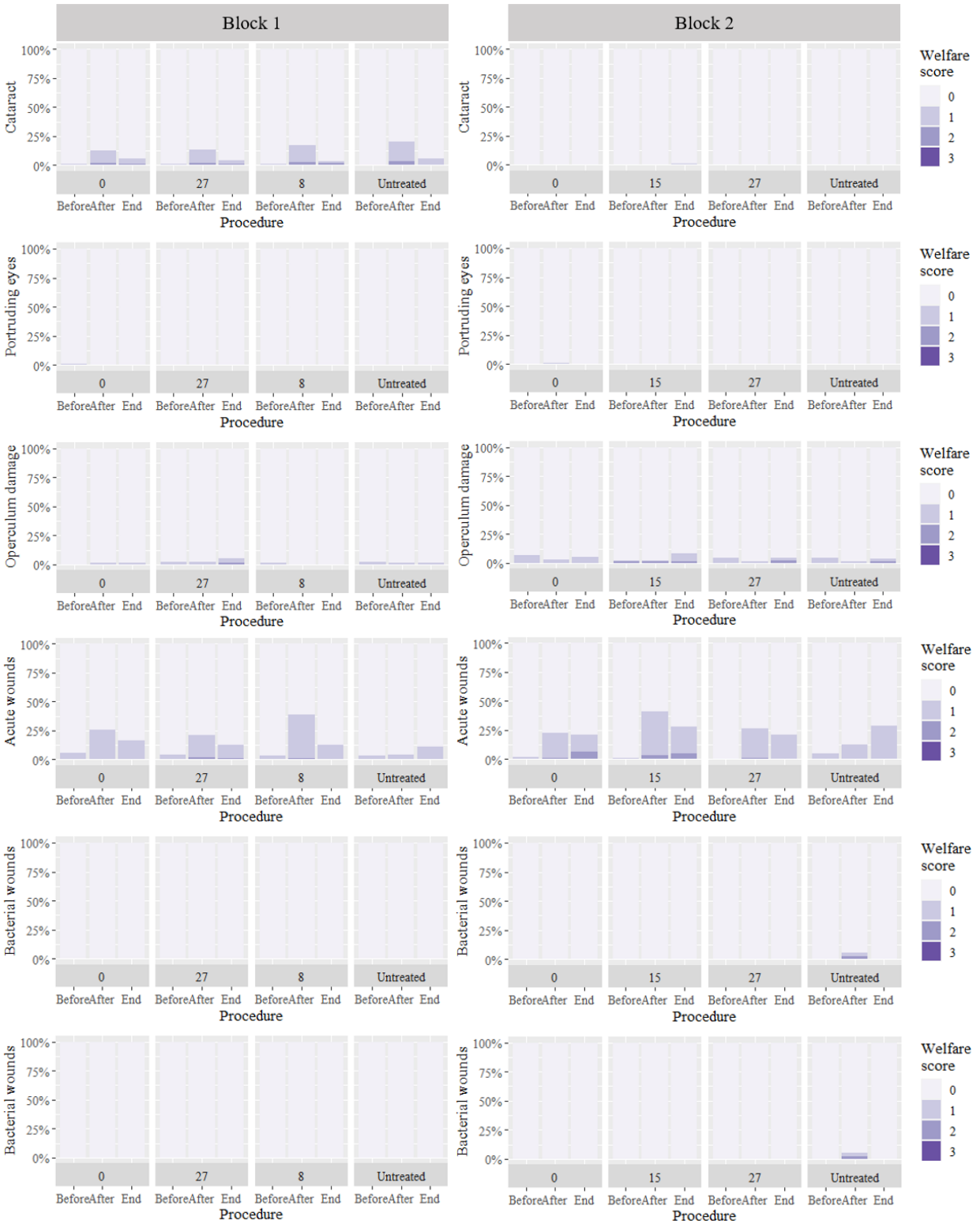
! = Unable to perform test due to negative values

5. Lice levels: before vs. after treatment

Comparison of delousing efficiency between groups right after treatment using lice levels at pit tagging as reference. Significance levels are p<0.05 (*), p<0.01 (**) and p<0.001(***), assessed by a Wilcoxon rank sum test.

Block	Lice stage	Compared treatments (°C)	Wilcoxon value	p-value
1	Sessile	0 – 27	3909	0.676
		0 – 8	3372	0.044*
		27 – 8	3654	0.096
	Preadult	0 – 27	3949	0.747
		0 – 8	3902	0.638
		27 – 8	4282	0.877
	Adult	0 – 27	4428	0.273
		0 – 8	4148	0.774
		27 – 8	3936	0.406
	Total	0 – 27	4385	0.332
		0 – 8	4329	0.419
		27 – 8	4184	0.895
2	Sessile	0 – 27	3530	0.065
		0 – 15	3187	0.692
		27 – 15	2787	0.172
	Preadult	0 – 27	2703	0.228
		0 – 15	2517	0.047*
		27 – 15	2211	< 0.001***
	Adult	0 – 27	3254	0.444
		0 – 15	3356	0.324
		27 – 15	3222	0.829
	Total	0 – 27	3560	0.065
		0 – 15	3396	0.266
		27 – 15	4131	< 0.001***

6. Plot of scores on remaining welfare parameters



Welfare scores before, right after treatment and at the end of trial based on remaining welfare parameters using the FISHWELL-scoring schema. Fish were kept at two holding temperatures (block 1: 8°C or block 2: 15°C) and had three treatment temperatures (0, 27 or 8/15°C).

7. Welfare: before treatment

Comparison of welfare scores (using the FISHWELL-scoring schema) between groups before treatment, assessed by a Wilcoxon rank sum test. Fish were kept at two holding temperatures (block 1: 8℃ and block 2: 15℃) and had three treatment temperatures 0, 27 or 8/15℃.

Welfare parameter	Block	Wilcox-value ≥	p-value ≥
Snout damage	1	3960	0.168
	2	!	!
Eye bleeding	1	3961	0.325
	2	10030	0.095
Skin bleeding	1	3608	0.093
	2	10808	0.120
Scale loss	1	4067	0.491
	2	3320	0.267
Fin bleeding	1	3809	0.449
	2	2929	0.530
Fin splitting	1	-	0.200 !!
	2	3533	0.088
Fin erosion	1	3923	0.349
	2	3166	0.639

! = All the individuals had score 0, thus no difference between groups.
!! = Adjusted p-value after Bonferroni correction.

8. Welfare: after treatment

Comparison of welfare scores (using the FISHWELL-scoring schema) between groups right after treatment. Fish were kept at two holding temperatures (block 1: 8°C and block 2: 15°C) and had three treatment temperatures 0, 27 or 8/15°C. Significance levels are p<0.05 (*), p<0.01 (**) and p<0.001, assessed by a Wilcoxon rank sum test.

Block	Welfare parameter	Compared treatments (°C)	Wilcox value	p-value
1	Snout damage	0 – 27	3571	0.141
		0 – 8	4600	0.088
		27 – 8	4303	0.834
	Eye bleeding	0 – 27	3752	0.339
		0 – 8	3792	0.403
		27 – 8	3660	0.073
	Skin bleeding	0 – 27	3653	0.195
		0 – 8	3738	0.284
		27 – 8	3547	0.026*
	Scale loss	0 – 27	3916	0.544
		0 – 8	4019	0.892
		27 – 8	4064	0.458
	Fin bleeding	0 – 27	3680	0.235
		0 – 8	4152	0.734
		27 – 8	3962	0.402
	Fin splitting	0 – 27	3527	0.065
		0 – 8	4221	0.561
		27 – 8	3901	0.268
	Fin erosion	0 – 27	4443	0.218
		0 – 8	3816	0.472
		27 – 8	4405	0.599
2	Snout damage	0 – 27	3042	NA
		0 – 15	3119	0.333
		27 – 15	3199	0.327
	Eye bleeding	0 – 27	3245	0.330
		0 – 15	3059	0.925
		27 – 15	3347	0.381

Skin bleeding	0 – 27	2918	0.640
	0 – 15	3085	0.988
	27 – 15	3026	0.622
Scale loss	0 – 27	3074	0.890
	0 – 15	3499	0.082
	27 – 15	3633	0.050
Fin bleeding	0 – 27	2747	0.245
	0 – 15	3240	0.533
	27 – 15	3031	0.622
Fin splitting	0 – 27	2803	0.366
	0 – 15	3094	0.961
	27 – 15	2924	0.386
Fin erosion	0 – 27	2930	0.670
	0 – 15	3207	0.634
	27 – 15	3179	0.947

9. Welfare: end of trial

9a) Treated vs. untreated groups

Comparison of welfare scores (using the FISHWELL-scoring schema) between treated and untreated groups at the end of the trial. Fish were kept at two holding temperatures (block 1: 8°C and block 2: 15°C) and had three treatment temperatures 0, 27 or 8/15°C. Significance levels are p<0.05 (*), p<0.01 (**) and p<0.001, assessed by a Wilcoxon rank sum test.

Welfare parameter	Block	Wilcox-value	p-value
Snout damage	1	11528	0.466
	2	9860	0.935
Eye bleeding	1	11401	0.353
	2	9892	0.958
Skin bleeding	1	13655	0.010*
	2	8911	0.134
Scale loss	1	12962	0.166
	2	10131	0.718
Fin bleeding	1	12425	0.662
	2	9382	0.428
Fin splitting	1	13848	0.022*
	2	10474	0.394
Fin erosion	1	12044	0.938
	2	9934	0.975

9b) Warm/cold water treatment vs. procedural control groups

Comparison of welfare scores (using the FISHWELL-scoring schema) between at the end of the trial. Fish were kept at two holding temperatures (block 1: 8℃ and block 2: 15℃) and had three treatment temperatures 0, 27 or 8/15℃. Significance levels are p<0.05 (*), p<0.01 (**) and p<0.001, assessed by a Wilcoxon rank sum test.

Block	Welfare parameter	Compared treatments (℃)	Wilcox value	p-value
1	Snout damage	0 – 27	4511	0.155
		0 – 8	3509	0.093
		27 – 8	4186	0.889
	Eye bleeding	0 – 27	3961	0.386
		0 – 8	3542	0.099
		27 – 8	4309	0.403
	Skin bleeding	0 – 27	4186	0.587
		0 – 8	4219	0.525
		27 – 8	4557	0.230
	Scale loss	0 – 27	3682	0.153
		0 – 8	3947	0.687
		27 – 8	3752	0.074
	Fin bleeding	0 – 27	3654	0.190
		0 – 8	4288	0.421
		27 – 8	4079	0.627
	Fin splitting	0 – 27	3538	0.089
		0 – 8	4064	0.961
		27 – 8	3743	0.122
	Fin erosion	0 – 27	4558	0.103
		0 – 8	3835	0.493
		27 – 8	4557	0.310
2	Snout damage	0 – 27	2963	0.743
		0 – 15	3449	0.139
		27 – 15	3485	0.205
	Eye bleeding	0 – 27	2932	0.499
		0 – 15	3110	0.831
		27 – 15	3077	0.583

Skin bleeding	0 – 27	2865	0.495
	0 – 15	3389	0.238
	27 – 15	3294	0.616
Scale loss	0 – 27	3311	0.252
	0 – 15	3065	0.951
	27 – 15	3421	0.285
Fin bleeding	0 – 27	2939	0.690
	0 – 15	3178	0.709
	27 – 15	3155	0.985
Fin splitting	0 – 27	3173	0.604
	0 – 15	3010	0.783
	27 – 15	3215	0.836
Fin erosion	0 – 27	3298	0.330
	0 – 15	2658	0.112
	27 – 15	2984	0.510