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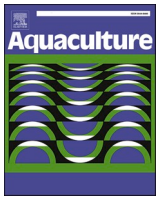

Long-term welfare effects of repeated warm water treatments on Atlantic salmon (*Salmo salar*)

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ABSTRACT

Warm water treatment has in recent years become widely used for ridding salmonids of sea lice in aquaculture although the consequences of the treatment for fish welfare are not adequately investigated. The objective of this study was to document potential long-term welfare effects of repeated warm water treatments on Atlantic salmon (*Salmo salar*). Five weeks after a baseline welfare indicator scoring, non-anaesthetised Atlantic salmon ($\bar{w}$ = 1379 ± 313 g, n = 332) were treated individually in a chamber with seawater at a temperature of 34 °C (warm water treatment) or 9 °C (control treatment) for 30 s. The treatment was repeated after 23–24 days. During the second treatment, a subset of the fish was video recorded for behavioural analysis. Seventeen to eighteen days after the second treatment, welfare indicators were again scored, and organ samples were taken for histopathological examination. The repeated warm water treatments resulted in a significantly increased prevalence and/or severity of scale losses, snout wounds, various eye problems, and active fin injuries as well as a significantly reduced specific growth rate. The fish displayed an immediate, strong behavioural reaction when exposed to warm water, which was probably the main cause of the detected injuries.

1. Introduction

Warm water treatment has in recent years become widely used for ridding salmonids of sea lice in aquaculture (Overton et al., 2018; Cerbule and Godfroid, 2020). There are various industrial warm water treatment systems, but the general operational procedure is to fast the fish for 2–7 days, crowd them in the sea cage, and pump them past a dewatering strainer into a treatment «chamber» where they are exposed to seawater at a temperature of 28–34 °C for 20–30 s (Holan et al., 2017; Noble et al., 2018; Haram, 2020). The consequences of warm water treatment for fish welfare are, however, not adequately investigated (Noble et al., 2018; Gismervik et al., 2020), and ever since this delousing method was taken into use, there have been anecdotal and scientific reports of high mortality (Overton et al., 2018; Walde et al., 2021) and various injuries (Gismervik et al., 2018; Poppe et al., 2018; Gismervik et al., 2019b, 2020) after treatment. The reported injuries include skin wounds, scale losses, fin injuries, degeneration of nasal mucosa, and bleedings in skin, brain, eyes, palate, gills, and thymuses.

Warm water treatment is not governed by the same stringent regulations as applied to medicinal treatments. Establishment of procedures and documentation of the treatment's effects on the fish has therefore been up to the aquaculture industry but are also tasks that need to be undertaken by associated academic and regulatory bodies. In general, temperature is one of the most important environmental factors for poikilothermic animals like salmonids, and it strongly affects for instance metabolism, growth rate, and the timing of life history events such as smoltification, migration, and spawning (Jonsson, 1991; Jonsson and L'Abée-Lund, 1993; Angilletta Jr et al., 2002). The optimum temperature range for Atlantic salmon (*Salmo salar*), defined as the range over which feeding occurs and there are no external signs of abnormal behaviour, is 6–20 °C (Elliott, 1981). Persistently higher temperatures or acute, large temperature changes appear to result in stress, reduced appetite, reduced growth rate, and/or mortality (Elliott, 1981; Elliott, 1991; Jobling, 1997). Salmonids respond to fluctuating water temperatures with short-term behavioural and physiological responses such as increases in activity level and oxygen consumption

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(Peterson and Anderson, 1969; Bellgraph et al., 2010; Folkedal et al., 2012a; Folkedal et al., 2012b). Water temperature changes also initiate behavioural and physiological acclimatisation processes that can last from days to weeks depending on the magnitude of the temperature change (Brett and Groves, 1979; Jobling, 1994).

Recently, controlled laboratory studies have been conducted to reveal whether exposure to water at the temperatures used in industrial warm water treatments against sea lice is painful for Atlantic salmon (Nilsson et al., 2019) and whether the thermal component of the treatment inflicts acute injuries on the fish (Moltumyr et al., 2021). In the study by Nilsson et al. (2019), Atlantic salmon ($\overline{w} = 234 \pm 52$ g) acclimated to seawater at a temperature of 8 °C were introduced individually into a tank containing seawater at one of 15 temperatures between 0 and 38 °C. Based on behavioural analysis, it was concluded that exposure to temperatures above ~28 °C was acutely aversive to the fish. In the study by Moltumyr et al. (2021), sedated Atlantic salmon ($\overline{w} = 1137 \pm 226$ g) were held individually in a soft bag and exposed to seawater at a temperature of 34 °C for 30 s. Welfare indicator scoring and histopathological examination of the fish revealed no evidence that the exposure led to any change in the prevalence of acute injuries except an increase in minor fin injuries. This increase may have been caused by a strong behavioural reaction displayed by the fish when exposed to the warm water. The negative results are supported by Kvåle (2020), who detected neither significant visible pathology nor histopathological changes in gill tissue of Atlantic salmon ($\overline{w} \sim 231$ g) one day or one week after exposure to seawater at a temperature of 34 °C for 30 s.

The salmon louse (*Lepeophtheirus salmonis*) develops rapidly at the water temperatures prevailing in summer, e.g. from copepodite to adult in 21 days at a water temperature of 15 °C (Hamre et al., 2019). As warm water treatment is only effective against the salmon louse when it is in mobile life stages (Grøntvedt et al., 2015; Roth, 2016), the treatment may have to be repeated after only a few weeks as any remaining sessile and newly settled lice continue to develop. Although there is now more knowledge about acute welfare effects of warm water treatment on salmonids (Nilsson et al., 2019; Kvåle, 2020; Moltumyr et al., 2021), the documentation of long-term welfare effects of repeated treatments is sparse. There is a possibility that potential injuries and/or other effects of the treatment may develop over time and not appear until the fish have been under clinical observation for days or weeks, as indicated for eye injuries and cataracts by Grøntvedt et al. (2015). If the treatment intervals are shorter than the healing time of the injuries, or the treatment inflicts permanent injuries on the fish, repeated treatments will lead to cumulative effects. In addition, frequent handling in connection with warm water treatments and other operations can cause chronic stress, weakened immune systems, and clinical disease outbreaks in fish (Barton et al., 1986; Maule et al., 1989; Wiik et al., 1989; Jacobson et al., 2003; Fast et al., 2008; Tort, 2011).

The objective of this study was to document potential long-term welfare effects of repeated warm water treatments on Atlantic salmon. Such documentation is important for fish health professionals and fish farmers when deciding which treatment strategy to adopt against sea lice. As a follow-up to the study by Moltumyr et al. (2021), in which the thermal component of the treatment was addressed, this study also takes the confinement and the behaviour of the fish into account.

2. Material and methods

2.1. Ethics

The study was approved by the Norwegian Food Safety Authority (ID No. 18041) and adhered to the Norwegian Animal Welfare Act (LOV-2009-06-19-97, LovData, 2009) and the Norwegian Regulation on Use of Animals in Experiments (FOR-2015-06-18-761, LovData, 2015).

2.2. Experimental animals and rearing conditions

The study was conducted with post-smolt Atlantic salmon (AquaGen® Atlantic QTL-innOva® PRIME strain, AquaGen, Inc., Trondheim, Norway) of mixed sexes reared at the Institute of Marine Research, Matre Research Station, Norway. The fish were first fed on the 22nd of February 2017 and were vaccinated with Aquavac® 6 vet. (Intervet International B.V., Boxmeer, The Netherlands) *on* the 11th of October 2017. On the 22nd of May 2018, the fish were transferred to a circular outdoor tank ($\varnothing = 7$ m, h = 1.5 m) with seawater ($S \sim 33$–34 ppt) at ambient temperature ($T \sim 7$–9 °C), where they were subjected to the prevailing natural photoperiod and fed standard commercial feed (Spirit Supreme, Skretting, Inc., Stavanger, Norway) in excess by automatic feeders during daytime. One week before the start of the study, the fish were transferred to a circular indoor tank ($\varnothing = 5$ m, h = 1.5 m, biomass ~29 kg m^{-3}) with the same water supply and feeding regime as in the outdoor tank. This indoor tank and an identical adjacent tank were alternately used as common stock tanks during the study (biomass ~22 kg m^{-3}). The fish were fasted for 2 days before and 1–2 days after all handling operations in the study.

At the start of the study, baseline welfare indicator scoring and PIT-tagging of the fish was conducted (Section 2.3.2). In addition to the scalpel incision wound from the PIT-tagging, the fish had varying extents of skin and snout wounds, skin bleedings, scale losses, and fin injuries (Section 3.4). To minimise background injuries and mortality, fish with severe injuries were euthanised and excluded from the study. The same applied to fish with signs of sexual maturation. To facilitate healing of the remaining fish, the salinity of the water in the stock tank was reduced to 25 ppt throughout the following acclimatisation and recovery period of five weeks. Three days before the first treatment, the salinity was returned to 34 ppt and kept at that concentration throughout the study. In the data analysis (Section 2.6), fish that had been subjected to handling mistakes or that could not be identified were excluded from the study. The baseline mean ± *SD* body weight and fork length of the 332 fish included in the study were 1379 ± 313 g and 49.9 ± 3.6 cm, respectively.

2.3. Experimental procedure

2.3.1. Timeline

The study was conducted in the period from the 4th of March to the 21st of May 2019 (i.e. 79 days) at Matre Research Station. The study comprised four operations:

1. Baseline welfare indicator scoring (including weight and length measurement) and PIT-tagging: 4th–5th of March.
2. First treatment: 8th–9th of April, i.e. 34–35 days after the baseline scoring.
3. Second treatment: 2nd–3rd of May, i.e. 23–24 days after the first treatment.
4. Final welfare indicator scoring (including weight and length measurement) and organ sampling: 20th–21st of May, i.e. 17–18 days after the second treatment.

A treatment interval of approximately three weeks was chosen because such short treatment intervals are relevant in the aquaculture industry during the peak season of the salmon louse (cf. Section 1).

2.3.2. Baseline welfare indicator scoring and PIT-tagging

Before the PIT-tagging, the water level in the stock tank was reduced and the fish were sedated (stage 1, Schoettger and Julin, 1967) by adding Aqui-S® vet. (isoeugenol 540 mg mL^{-1}, Scan Aqua Inc., Årnes, Norway) to a concentration of 4–9 mL 1000 L^{-1} of seawater. Then, groups of about five fish at a time were dip-netted from the stock tank and transferred to a cart where they were anaesthetised (stage 4, Schoettger and Julin, 1967) with Finquel® vet. (tricaine

methanesulfonate 1000 mg g^{-1}, Scan Aqua, Inc., Årnes, Norway) at a concentration of 100 mg L^{-1} of seawater. The fish were thereafter placed onto a V-shaped board covered with a wet cloth and tagged with PIT-tags in the abdominal cavity through a scalpel incision. PIT-tags of equal size but with two different lengths of their number sequences were mixed approximately half of each in a container and were randomly selected to assign the fish to a warm water group and a procedural control group and to facilitate sorting of the fish into these groups during the subsequent treatment. The internal PIT-tags also ensured that the evaluators were blinded to information on treatment group during the later welfare indicator scorings.

After the PIT-tagging, the fish were weighed to the nearest gram and their fork lengths were measured to the nearest 0.5 cm. Then, the fish were transferred to rectangular plastic trays containing seawater from the stock tank for scoring of welfare indicators. The welfare indicators of each fish were scored by one of two evaluators. The welfare indicator scoring schema used was based on Noble et al. (2018) but also included additional welfare indicators that could be relevant to warm water treatment (Poppe et al., 2018). The whole body of the fish was visually inspected for exterior, macroscopic deformities, injuries, and signs of disease. When scoring, paired organs and fins were given one common score based on the most severely affected organ and fin, respectively. Vertebral, opercular, and jaw deformities, skin wounds, skin bleedings, scale losses, protruding scales, snout wounds, eye injuries, eye bleedings, eye opacity, exophthalmos (i.e. protruding eyes), gill injuries, gill bleedings, gill paleness, gill spots (i.e. white and necrotic areas), and fin injuries (all fins except the adipose fin) were scored on a scale from 0 to 3, denoting no, mild, moderate, and severe, respectively. For eye opacity, 0 = no, 1 ≤ 25%, 2 = 25–75%, and 3 ≥ 75% opaque covering of the eye. Fin injuries (i.e. fin splits and fin erosion) were scored depending on how far towards the fin basis the splitting and/or eroding had progressed, where 0 = no splits/erosion, 1 = shallow split(s)/erosion, 2 = deeper split(s)/erosion, and 3 = split(s)/erosion to the fin base. Only active fin injuries (i.e. with external or internal bleedings) were scored. Mucus on skin and gills were recorded if in surplus or deficit, and bleedings in the nasal pits, palate, and thymuses were recorded if present.

2.3.3. First treatment

In the first treatment, fish were randomly and individually dip-netted from the common stock tank while the water level in the tank was gradually lowered to facilitate the netting. This meant that the fish in both treatment groups were crowded for 0–6 h before the treatment. The crowding intensity varied somewhat but was generally low and did not cause the fish to become exhausted or collide with the tank walls or other fish. While in the dip net, the PIT-tags of the fish were read for recording of which treatment group the fish belonged to. Beyond this, no weight or length measurement or welfare indicator scoring of the fish was conducted to avoid imposing strains on the fish that were not directly related to the treatments. Thereafter, the fish were transferred to a rectangular, transparent plastic bin (Fig. 1a) that was immerged for 30 s in a tank (Fig. 1b) containing seawater at a temperature of either 34 °C for warm water treatment or 9 °C for control treatment.

The rationale for treating the fish in a hard bin, was to mimic the stainless steel treatment «chambers» that are currently used in industrial warm water treatment systems (Grøntvedt et al., 2015; Roth, 2016) and to reflect the physical strains that the fish experience in such chambers. In accordance with this rationale, the fish were not anaesthetised before treatment. Five holes were drilled in the bottom of the bin to let water flow in and out. The bottom and the lid of the bin were padded with non-slip fabric to prevent injuries on the fish due to irregular structures, while the plain sides of the bin were left non-padded to enable observation of the fish during treatment.

The water in the treatment tanks originated from the same source as the water in the stock tank. The water in the warm water treatment tank was heated by an EVO Swimming Pool Heater (Elecro Engineering, Ltd., Stevenage, UK) and was circulated through the heater by means of a pump placed on the bottom of the tank. The temperature and oxygen saturation of the water were checked before each fish was introduced in the warm water treatment tank and regularly in the control treatment tank. The warm water was continuously aerated by being moved around by the water pump and being manually stirred and lifted with the treatment bin. The mean temperature ± *SD* was 33.5 ± 0.3 °C ($n = 148$) in the warm water and 8.5 ± 0.0 °C ($n = 5$) in the control water (176 T2 temperature data logger, Testo, Inc., Lenzkirch, Germany). The mean oxygen saturation ± *SD* was 125 ± 3% ($n = 33$) in the warm water and always between 95 and 105% in the control water (Handy Polaris 2 oxygen meter, OxyGuard International, Inc., Farum, Denmark).

After treatment, the fish were transferred via the dip net to the other stock tank. The fish were checked upon continuously during the first three hours after treatment and thereafter received daily supervision.

2.3.4. Second treatment

In the second treatment, the same procedure as in the first treatment (Section 2.3.3) was followed, and each fish got the same treatment as the first time. The mean temperature ± *SD* was 33.7 ± 0.2 °C ($n = 149$) in the warm water and 9.2 ± 0.8 °C ($n = 5$) in the control water. The mean oxygen saturation ± *SD* was 106 ± 5% ($n = 145$) in the warm water and 98 ± 1 °C ($n = 11$) in the control water. A subset of the exposures was video recorded from above by means of a hand-held camera (Tough TG-5, Olympus Norge, Inc., Asker, Norway) for behavioural analysis. To enable this, 44 fish were treated without padding in the lid of the treatment chamber.

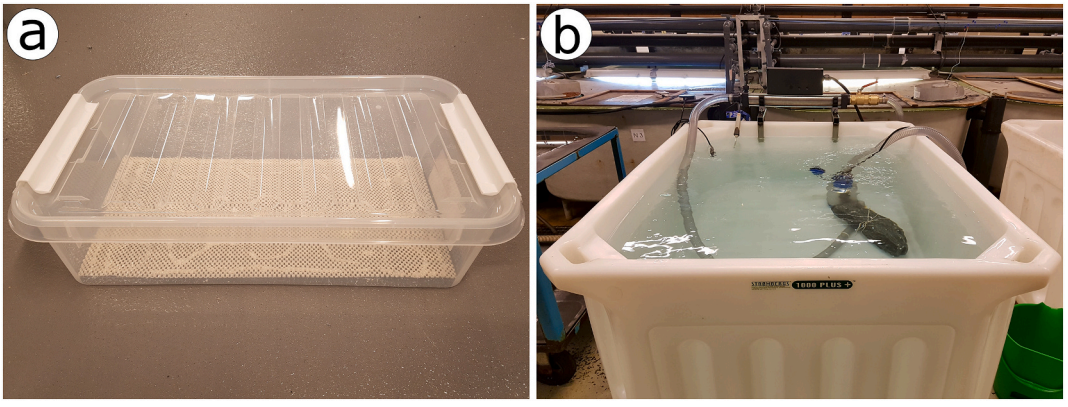

Fig. 1. a) Bin used as treatment chamber (L65 × W35 × H15 cm^3), here shown without padding in the lid. b) Warm water treatment tank ($V = 1000$ L) with heater and water pump.

2.3.5. Final welfare indicator scoring and organ sampling

In the final welfare indicator scoring, the same procedure as in the baseline welfare indicator scoring (Section 2.3.2) was followed, and the scoring was conducted by the same two evaluators. Fish with one or two punctured eyes (i.e. eye injuries score 3) were not given scores on eye bleedings or eye opacity as these welfare indicators could not be assessed as intended due to the puncture. This applied to 17 fish in the warm water group and 9 fish in the control group. After the final scoring, the fish were euthanised with Finquel® vet. at a concentration of 500 mg L^{-1} of seawater. The rationale for not euthanising the fish before the final scoring, was to avoid potential changes in welfare indicators (e.g. gill paleness and amount of skin and gill mucus).

After euthanisation, organ samples were collected from 18 fish, of which 13 from the warm water group and 5 from the control group. The samples were taken from the left side of the fish and included skin, gill, pseudobranch, and thymus. The skin samples (L2 × W0.5 × H1 cm^3) were taken across the lateral line ventrally to the dorsal fin, and the gill samples were taken from the second gill arch. All samples were fixed in a 4% neutral buffered formaldehyde solution and stored at room temperature ($T \sim 21$ °C) awaiting processing. In addition, a part of the scull roof on 23 fish, of which 13 from the warm water group and 10 from the control group, was removed to expose the brain and macroscopically check for cerebral haemorrhage.

2.4. Histopathological examination

The fixed organ samples were prepared for histopathological examination by processing in a Citadel 2000 Tissue Processor (Thermo Fisher Scientific, Inc., Massachusetts, USA), followed by paraffin wax embedding, slicing, and deparaffinisation at the Norwegian College of Fishery Science in Tromsø, Norway. Subsequently, the sections of gill, pseudobranch, and thymus were stained with hematoxylin and eosin (HE) to facilitate detection of histopathological changes. As the carbohydrate composition of epithelial mucus layers has been shown to change with stress and environmental conditions (Zaccone et al., 2001), an Alcian blue – periodic acid-Schiff (AB–PAS) staining was conducted on the sections of skin and gill to allow categorisation and counting of goblet cells producing acidic and neutral glycoproteins, respectively (Fletcher et al., 1976). After staining, the tissue sections were examined by means of a light microscope. The two evaluators were blinded to treatment information on the fish. The percentage of affected tissue was determined by assessment of the entire tissue section and was graded on a scale from 0 to 3, where 0 ≤ 5%, 1 = 5–10%, 2 = 10–50%, and 3 ≥ 50% (George et al., 2016). Counting of goblet cells was conducted in an area of 0.13 mm^2 of each tissue section (i.e. the entire field of view through a 40× objective lens), and the sections were oriented so that areas with a representative density of mucus cells for each section were examined. Due to processing and sectioning issues and difficulties finding the relevant tissues in the sections, the number of examined samples varies between organs and are given in the results.

2.5. Behavioural analysis

The behavioural analysis was based on video recordings of sufficient quality from the second treatment and included 39 fish, of which 19 fish in the warm water group and 20 fish in the control group. As the treatment chamber was small relative to the fish size, it limited the fish's behavioural repertoire: it allowed the fish to move and turn in the horizontal plane but barely allowed any movement in the vertical plane. To obtain an objective, quantifiable measurement of the fish's behaviour, the video recordings from above the treatment chamber were analysed with regard to the number of head direction changes the fish performed during treatment, or more specifically the number of times the fish changed the direction of their head relative to their anteroposterior axis during the exposure time of 30 s. The number of head direction changes was counted manually for each fish.

2.6. Data analysis

At the start of the data analysis, fish that had been subjected to handling mistakes during the study (e.g. escaped from the treatment chamber) and fish with malfunctioning or misread PIT-tags (and thus unknown identities) were excluded from the data set. Fisher's Exact Test for Count Data was used to test whether there was no difference in mortality between fish in the warm water group and the control group in the period from the start of the first treatment to the end of the study (*fisher.test*, R Core Team 2019). The condition factor of the fish (K) was calculated using Fulton's formula (Ricker, 1975), and the specific growth rate of the fish (SGR) was calculated according to Schmalhausen (1926) as cited in Ricker (1979). As the fish were not weighed at the treatments, their specific growth rates had to be calculated over the period of 79 days from the start of the baseline welfare indicator scoring to the end of the final welfare indicator scoring. Welch Two Sample *t*-Test (*t.test*, R Core Team 2019) was used to test whether there were no differences in mean body weight, fork length, condition factor, or specific growth rate between fish in the two treatment groups. Generalised Linear Model (GLM) with quasi-Poisson errors to adjust for overdispersion (*glm, family = quasipoisson*, R Core Team 2019) was used to test whether there was no difference in the mean number of head direction changes performed by video recorded fish in the treatment groups during the second treatment. GLM with quasi-Poisson errors was also used to test whether there was no effect from treatment on the number of goblet cells producing acidic and neutral glycoproteins, respectively, in the tissue sections of skin and gill at the time of the final welfare indicator scoring. GLM with binomial errors (*glm, family = binomial*, R Core Team 2019) was used to test whether there were no effects on the welfare scores (score ≥ 1, score ≥ 2, and score = 3) from evaluator (E1 or E2), lid padding (present or absent), or treatment (warm water or control). GLM with binomial errors was also used to test whether there was no effect from treatment on the presence of histopathological changes in gill, pseudobranch, and thymus at the time of the final welfare indicator scoring. In all statistical tests, the significance level was set at 0.05.

3. Results

3.1. Mortality

After the first treatment, one fish in the warm water group occasionally spun around on the grate at the bottom of the stock tank. This fish died the same evening, seemingly from circulatory failure (autopsied). One fish from each treatment group were euthanised at the second treatment due to punctured eyes and consequent blindness. Two fish in the warm water group died in the period between the second treatment and the final welfare indicator scoring. The causes of these deaths are unknown. Hence, there were 4dead and 149 alive fish in the warm water group and 1 dead and 178 alive fish in the control group (difference in mortality: 4 out of 153 vs. 1 out of 179, $p = 0.186$).

3.2. Growth

In the baseline welfare indicator scoring, there were no significant differences in mean body weight or fork length between fish in the warm water group ($n = 149$) and the control group ($n = 178$) (Fig. 2a, b), but fish in the warm water group had on average a higher condition factor (± *SE*) than fish in the control group ($\overline{K} = 1.10 \pm 0.005$ vs. $\overline{K} = 1.08 \pm 0.005$, $t = 2.78$, $p = 0.006$) (Fig. 2c). In the final welfare indicator scoring, however, fish in the warm water group had significantly lower mean body weight ($\overline{w} = 1547 \pm 30$ g vs. $\overline{w} = 1675 \pm 31$ g, $t = -3.0$, $p < 0.001$) and shorter mean fork length ($\overline{l} = 51.9 \pm 0.3$ cm vs. $\overline{l} = 53.3 \pm 0.3$ cm, $t = 3.2$, $p = 0.002$) than fish in the control group (Fig. 2a, b), while the mean condition factor of the fish was similar in both treatment

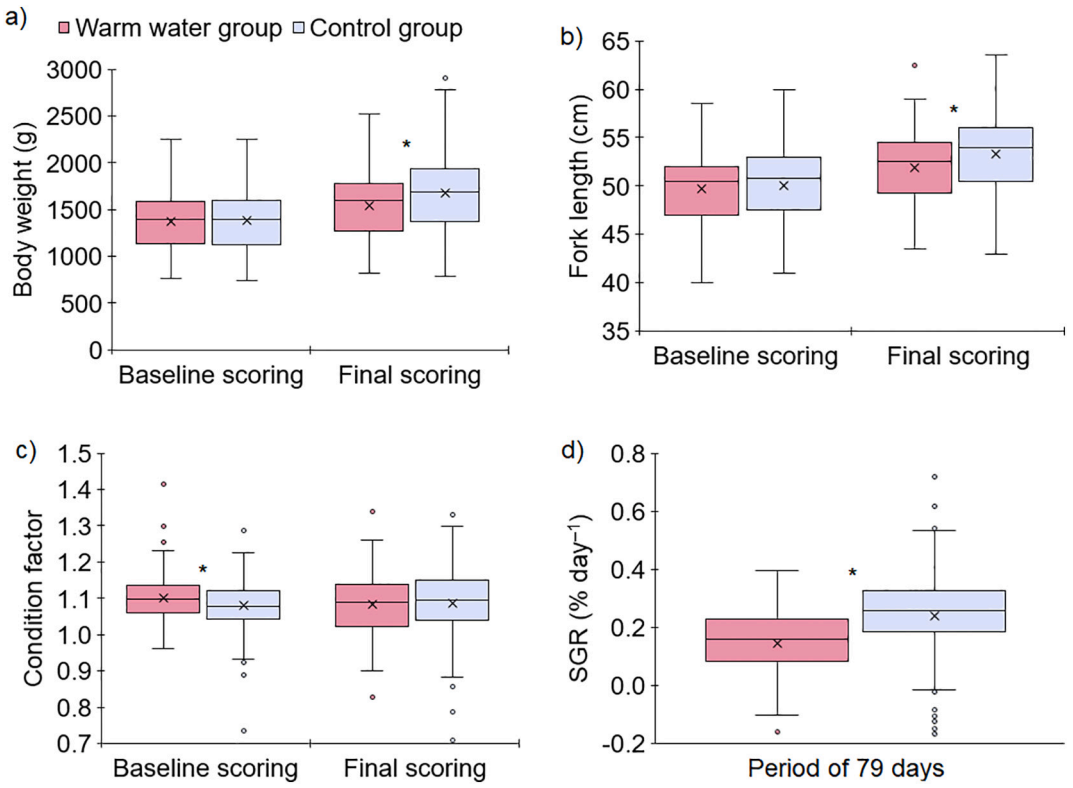

Fig. 2. Boxplots of a) body weight, b) fork length, and c) condition factor for fish in the warm water group ($n = 149$) and the control group ($n = 178$) in the baseline and final welfare indicator scorings, and d) specific growth rate for fish in the same treatment groups in the period of 79 days from the start of the baseline scoring to the end of the final scoring. Boxplot explanation: cross = mean, middle line of box = median, bottom line of box = median of 1st quartile, top line of box = median of 3rd quartile, bottom whisker = lowest value within 1.5 times the interquartile range, top whisker = highest value within 1.5 times the interquartile range, circles = outliers, and asterisk = statistical significance.

groups ($\overline{K} = 1.1 \pm 0.0$, $t = 0.29$, $p = 0.776$) (Fig. 2c). In the period of 79 days from the baseline to the final scoring, fish in the warm water group had a significantly lower mean specific growth rate than fish in the control group ($\overline{SGR} = 0.15 \pm 0.01\%$ day^{-1} vs. $\overline{SGR} = 0.24 \pm 0.01\%$ day^{-1}, $t = -6.4$, $p < 0.001$) (Fig. 2d). Including baseline condition factor as a possible explanatory parameter in a GLM comparing the specific growth rates of fish in the two treatment groups, revealed no significant effect from baseline condition factor on specific growth rate ($t = 1.4$, $p \geq 0.177$) and confirmed the negative effect of the warm water treatments ($t = -6.4$, $p < 0.001$).

3.3. Behaviour

The fish in the warm water group displayed an immediate, strong behavioural reaction when exposed to the warm water in the treatment chamber, with vigorous wriggling and frantic attempts to escape (Suppl. Mat.: Video 1a). During this behavioural reaction, several fish (including 3 out of 19 video recorded fish in the warm water group) collided so violently with the walls of the treatment chamber that they fainted and ended up lying sideways on the bottom of the chamber for some seconds (3–11 s in the video recorded fish) before resuming the wriggling behaviour. In contrast, the fish in the control group behaved more calmly and seemingly systematically tried to find a way out of the chamber (Suppl. Mat.: Video 1b). When that failed, many of the fish sat down on the bottom of the chamber until the treatment time expired. The distinct difference in behaviour between fish in the two treatment groups is quantified by a significantly higher mean number of head direction changes performed by video recorded fish in the warm water group than in the control group (32.1 ± 1.4 ($n = 16$ as the 3 fish that fainted were excluded from the analysis) vs. 10.1 ± 0.9 ($n = 20$), $t = 12.1$, $p < 0.001$) (Fig. 3). When released into the stock tank after

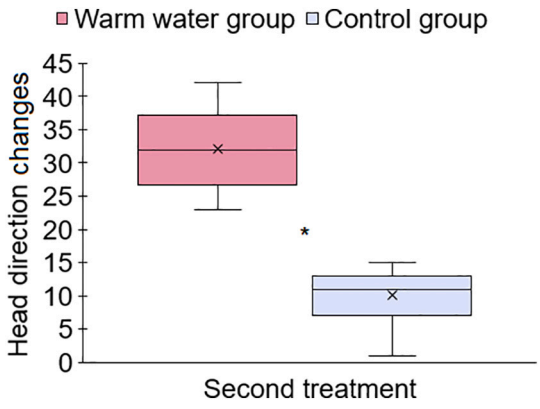

Fig. 3. Number of head direction changes performed by video recorded fish in the warm water group ($T \sim 34$ °C, $n = 16$) and the control group ($T \sim 9$ °C, $n = 20$) during the second treatment ($t = 30$ s). Boxplot explanation is given in the caption of Fig. 2.

treatment, the fish gradually returned to their normal shoaling behaviour. Several fish were observed with transient dark snouts and/or exophthalmos, but due to the lack of external tags, these fish could not be identified to treatment group.

3.4. Welfare indicator scores

The results of the welfare indicator scorings are given in Table 1. The prevalence of deformities was 1–3% in the treatment groups in both the baseline and the final welfare indicator scoring but is not presented in

Table 1
Prevalence (%) and severity of welfare indicators for fish in the warm water group and the control group in the baseline and final welfare indicator scorings. Scores: 1 = mild, 2 = moderate, and 3 = severe. Significantly higher prevalence ($p < 0.05$) when comparing welfare indicator scores in the two treatment groups at each scoring, is indicated by *. Significant changes in prevalence ($p < 0.05$) when comparing welfare indicator scores in each treatment group at the two scorings, are indicated by ↑ if higher and ↓ if lower. Warm water group: $n = 149$ and control group: $n = 178$ except for eye bleedings and eye opacity in the final welfare indicator scoring, where warm water group: $n = 132$ and control group: $n = 169$.

Welfare indicator scoring		Baseline						Final					
Treatment group		Warm water			Control			Warm water			Control		
Welfare indicator scores		1 + 2 + 3	2 + 3	3	1 + 2 + 3	2 + 3	3	1 + 2 + 3	2 + 3	3	1 + 2 + 3	2 + 3	3
Skin	Skin wounds	21	3	0	22	2	0	6↓	2	0	3↓	0	0
	Skin bleedings	73	3	0	79	3	0	60	1	0	54	1	0
	Skin mucus	0			0			0			0		
	Scale losses	100	79	0	99	75	0	100	42↓*	0	99	19↓	0
	Protruding scales	0	0	0	0	0	0	0	0	0	0	0	0
Snout	Snout wounds	7	1	0	12	1	0	99↑	93↑*	14↑*	99↑	53↑	1
	Nasal pit bleedings	0			0			0			0		
	Palate bleedings	1			0			0			0		
Eyes	Eye injuries	0	0	0	0	0	0	12↑*	12↑*	11↑*	5↑	5↑	5↑
	Eye bleedings	5	0	0	3	0	0	28↑*	5↑	1	15↑	1	1
	Eye opacity	10	0	0	10	2	0	56↑	14↑*	3	49↑	6	0
	Exophthalmos	0	0	0	1	0	0	3	2	2	1	0	0
Gills	Gill injuries	1	0	0	0	0	0	4	0	0	2	1	0
	Gill bleedings	1	0	0	1	1	0	0	0	0	1	0	0
	Gill paleness	1	0	0	4	0	0	1	0	0	0	0	0
	Gill spots	10	0	0	15	0	0	7	1	0	3↓	1	1
	Gill mucus	0			0			0			0		
	Thymic bleedings	24			17			9↓			24*		
Fins	Dorsal fin injuries	50	41	1	59	46	1	86↑*	82↑*	9*	70↑	57	3
	Pectoral fin injuries	81	79	4	78	74	3	79*	77*	6	67↓	62↓	4
	Pelvic fin injuries	91	81	1	85	78	1	99↑	97↑	5	98↑	92↑	0
	Anal fin injuries	87	64	1	88	65	0	99↑	93↑	7	99↑	87↑	3
	Caudal fin injuries	66	43	3	66	38	4	100↑	85↑*	5	82↑	34	2

the table.

The prevalence of skin wounds (21–22%), skin bleedings (73–79%), and scale losses (99–100%) was high in the treatment groups in the baseline scoring. Most of the scale losses (75–79%) were of moderate severity (score 2). In the final scoring, there was a general trend of improved skin condition, but the prevalence of scale losses of moderate severity was significantly higher in the warm water group (42%) than in the control group (19%). No protruding scales or abnormal amounts of skin mucus were detected on any of the fish in any of the scorings.

The prevalence of snout wounds was 7–12% in the treatment groups in the baseline scoring. In the final scoring, the prevalence (99%) and severity of snout wounds in the treatment groups were significantly higher than in the baseline scoring. The severity of the snout wounds (score 2 + 3 and score 3) was also significantly higher in the warm water group than in the control group. There were few detections of palate bleedings and no detections of nasal pit bleedings on any of the fish in any of the scorings.

The prevalence of eye bleedings was 3–5% and the prevalence of eye opacity was 10% in the treatment groups in the baseline scoring. Furthermore, there were no detections of eye injuries and only one detection of exophthalmos on the fish. In the final scoring, however, the prevalence of eye injuries (5–12%), eye bleedings (15–28%), and eye opacity (49–56%) was significantly higher in both treatment groups and significantly higher in the warm water group than in the control group. The severity of eye injuries (score 2 + 3 and score 3) and eye opacity (score 2 + 3) was also significantly higher in the warm water group than in the control group.

The prevalence of gill injuries, gill bleedings, and gill paleness was 1–4% in the treatment groups in both scorings. No abnormal amounts of gill mucus were detected on any of the fish. The prevalence of gill spots was 10–15% and the prevalence of thymic bleedings was 17–24% in the treatment groups in the baseline scoring. In the final scoring, however, the prevalence of gill spots in the control group (3%) and the prevalence of thymic bleedings in the warm water group (9%) were significantly lower. Also, the prevalence of thymic bleedings was significantly higher in the control group (24%) than in the warm water group.

The prevalence of fin injuries was 50–100% in the treatment groups in both scorings, and the injuries were mainly of moderate (score 2) severity. The prevalence of fin injuries was significantly higher in both treatment groups in the final scoring than in the baseline scoring, except for the prevalence of pectoral fin injuries, which was not significantly altered in the warm water group but was significantly lower in the control group. The severity of the injuries on the dorsal, pectoral, and caudal fins in the final scoring was significantly higher in the warm water group than in the control group.

No cerebral haemorrhage was detected in any of the examined fish.

3.5. Histopathological changes

At the time of the final welfare indicator scoring (17–18 days after the second treatment), there were no significant differences in histopathological changes in gill, pseudobranch, or thymus between examined fish in the warm water group and the control group ($z \sim 0.0$, $p > 0.997$) (Table 2). Mild histopathological changes (all score 1) were detected in three different fish from the warm water group. The changes were small lamellar aneurysms in 2 out of 10 gill sections (Fig. 4a) and mild focal haemorrhages in 1 out of 9 thymus sections (Fig. 4b). There were no significant differences in the number of goblet cells producing

Table 2
Histopathological changes in gill, pseudobranch, and thymus from examined fish in the warm water group ($T \sim 34$ °C) and the control group ($T \sim 9$ °C) at the time of the final welfare indicator scoring. As no histopathological changes affected more than 10% of the tissue sections, only scores 0 and 1 are presented in the table.

Treatment group	Warm water			Control		
Organ	Score 0	Score 1	*n*	Score 0	Score 1	*n*
Gill	8	2	10	4	0	4
Pseudobranch	8	0	8	4	0	4
Thymus	8	1	9	4	0	4

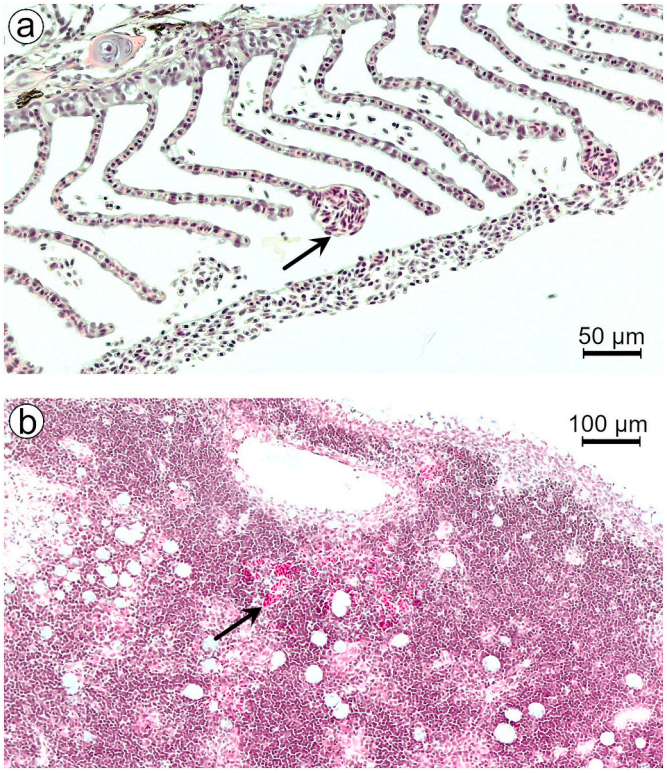

Fig. 4. Examples of histopathological changes (all score 1) in HE-stained tissue sections from fish in the warm water group at the time of the final welfare indicator scoring. a) Small lamellar aneurysm (arrow) in gill (20× objective lens). b) Mild focal haemorrhage (arrow) in thymus (10× objective lens).

acidic and neutral glycoproteins, respectively, in the tissue sections of skin and gill between examined fish in the warm water group and the control group ($t > 1.4$, $p > 0.183$) (Table 3).

4. Discussion

4.1. Mortality

No significant difference in mortality between fish in the two treatment groups was found in this study. The mortality in the warm water group was 4 out of 153 fish, i.e. 2.6%. In a net pen containing 200 k fish, 2.6% is equivalent to ~5200 fish. It should be recalled that fish with severe injuries and signs of sexual maturation were excluded before the first treatment in this study, probably leaving a relatively robust fish group to be treated. Treatment of fish with various challenges or underlying pathologies, e.g. amoebic gill disease (AGD), cardiomyopathy syndrome (CMS), or heart and skeletal muscle disease (HSMD), can result in considerably higher mortality (Grøntvedt et al., 2015; Gismervik et al., 2018). As several of the remaining fish in this study had serious (score 3) eye injuries and snout wounds in the final welfare indicator scoring, it is possible that some of them would eventually have ended up as delayed mortalities after the treatments or after an additional treatment that could have been required during industrial salmon production.

4.2. Growth

The mean specific growth rate of 0.24% day^{-1} for fish in the control group in this study was considerably lower than the expected growth rate of 0.65–0.68% day^{-1} for Atlantic salmon of similar baseline weight at a water temperature of 8 °C (Skretting, 2012). Nevertheless, the mean specific growth rate of fish in the warm water group (0.15% day^{-1}) was significantly lower than that of fish in the control group. The period of 79 days over which the specific growth rates were calculated, included a baseline scoring and PIT-tagging of 2 days and an acclimation and recovery period of 33 days where the fish in both treatment groups were kept in a common stock tank. It is therefore reasonable to assume that the difference in growth between the treatment groups arose during the period of 44 days from the start of the first treatment to the end of the final welfare indicator scoring, and that the actual negative effect of the warm water treatments on the specific growth rate of the fish was larger than shown here.

The lower than expected specific growth rates of fish in both treatment groups may have been related to the amount of stress and injuries the fish experienced during the study. Crowding and handling are inherently stressful (Barton and Iwama, 1991; Barton, 2002; Basrur et al., 2010), and fish acclimated to a specific temperature or range of temperatures experience a heat shock when exposed to a rapid and large increase in water temperature (DuBeau et al., 1998; Currie et al., 2000; Nakano et al., 2014). The PIT-tagging, welfare indicator scorings, and treatment operations in this study thus represented acute stressors that, due to the heat shock, were particularly potent for fish in the warm water group. In addition, injuries that were inflicted on the fish during these operations may have represented chronic stressors during or throughout the study. In the final welfare indicator scoring, injuries were most prevalent among fish in the warm water group, probably mainly due to the behavioural reaction these fish displayed during the treatments and not the warm water per se (cf. Kvåle, 2020; Moltumyr et al., 2021). It is documented that intense acute and chronic stressors have appetite-reducing effects on fish (Carr, 2002; Bernier, 2006; Pankhurst et al., 2008; Madaro et al., 2015; Volkoff and Rønnestad, 2020). Thus, one reason for the growth difference between fish in the two treatment groups may be that fish in the warm water group experienced more stress than fish in the control group, resulting in more strongly and/or more persistently reduced appetite. Another contributing factor may be that fish with various eye problems, of which there were significantly most in the warm water group, had reduced vision and consequently reduced ability to see the feed.

4.3. Behaviour

The behaviour of the fish in the control group during the treatments resembled that of fish caught in pots, where after some initial escape attempts, the fish typically either swims around in the available space inside the pot or settles down and rests within it (Anders et al., 2017; Meintzer et al., 2017). The fish in the warm water group, however, displayed an immediate, strong behavioural reaction when exposed to

Table 3
Number of goblet cells producing acidic and neutral glycoproteins, respectively, in tissue sections of skin and gill from fish in the warm water group and the control group at the time of the final welfare indicator scoring.

	Treatment group	Warm water				Control			
Organ	Goblet cell glycoproteins	Total	Mean	*SE*	*n*	Total	Mean	*SE*	*n*
Skin	Acidic	188	18.8	6.6	10	77	15.4	8.5	5
	Neutral	245	24.5	7.8	10	111	22.2	8.8	5
Gill	Acidic	206	22.9	1.7	9	105	21.0	1.6	5
	Neutral	18	2.0	0.5	9	4	0.8	0.6	5

the warm water in the treatment chamber. This is in accordance with previous reports from similar warm water treatments of salmonids (Roth, 2016; Moltumyr et al., 2021). When exposed to water at this high temperatures, salmonids display abnormal behaviours such as frequent direction changes and surface breaks, high swimming speeds, and collisions with enclosure walls, equipment, and other fish (Elliott, 1991; Elliott and Elliott, 1995; Ineno et al., 2005; Roth, 2016; Nilsson et al., 2019). Nilsson et al. (2019) suggest that this behavioural reaction indicates nociception or pain, and it has been discussed whether the reaction is the cause of many of the injuries detected on fish after industrial warm water treatments (Gismervik et al., 2018; Moltumyr et al., 2021).

Such bouts of activity as the behavioural reaction represented, involves an anaerobic, high power output from white locomotory muscles. This depletes endogenous energy stores in the form of adenosine triphosphate, phosphocreatine, and glycogen (Dobson and Hochachka, 1987; Kieffer, 2000) and results in an accumulation of lactate (Wood, 1991; Wang et al., 1994; Milligan, 1996). The activity can only be maintained for short periods of time and end in fatigue (Beamish, 1978; Wood, 1991). Following an activity bout, the recovery of adenosine triphosphate and phosphocreatine is generally quite rapid and usually occurs within one hour (Milligan and Wood, 1986; Wang et al., 1994). The removal of lactate and re-synthesis of glycogen are, however, much slower and have been reported to require up to 24 h in some species (Black et al., 1962; Wardle, 1978; Milligan and Wood, 1986). Thus, the fish in the warm water group probably needed longer time to recover from the treatments than the fish in the control group.

After each treatment, several fish were observed with transient dark snouts and/or exophthalmos in the stock tank, but due to the lack of external tags, these fish could not be identified to treatment group. Dark snouts were also observed in the study by Kvåle (2020), where warm water treated fish and control fish were kept in separate tanks: At sampling one day after treatment, dark snouts were observed on most of the warm water treated fish. At sampling one week after treatment, dark snouts were not observed on any of the fish. The dark snouts and exophthalmos observed in the present study may have been caused by the warm water, stress, or mechanical impact from the dip net or collisions with the walls of the treatment chamber. As the treatment chamber was small relative to the fish size, it did not allow the fish to reach high swimming speeds before colliding with the chamber walls. This reduced the impact force in the collisions. Nevertheless, some fish collided so violently with the chamber walls that they fainted.

The behaviour of the fish is also a challenge during industrial warm water treatments. The fish jump and bounce on the dewatering strainer and make swimming bursts in the treatment chambers that cause them to collide with other fish, enclosure walls, and equipment (Roth, 2016). Use of anaesthetics before treatment can make the fish calmer and dampen their behavioural reaction throughout the treatment procedure and thus reduce behaviour-related injuries (Folkedal et al., 2021). In industrial warm water treatments, however, use of anaesthetics is challenging due to the large numbers of fish and will demand a rethinking of existing methodologies and constructions at all procedural levels (Folkedal et al., 2021). It should also be mentioned that industrial warm water treatments currently cannot be conducted without associated handling operations and that the mechanical part of the treatment (crowding, pumping, straining, and transportation through the treatment systems) in itself can cause wounds, tears, and clamp and stroke injuries (Grøntvedt et al., 2015; Roth, 2016; Noble et al., 2018) regardless of whether anaesthetics are used.

4.4. Injuries

In the baseline welfare indicator scoring, skin bleedings, scale losses, and active fin injuries were dominating among fish in both treatment groups. The prevalence of skin wounds, snout wounds, eye opacity, gill spots, and thymic bleedings was also relatively high. Most of these injuries indicate mechanical impact on the fish and may have been inflicted during transfer of the fish from the outdoor tank to the indoor tank one week before the start of the study. In the control group, skin wounds, scale losses, gill spots, and pectoral fin injuries were significantly more prevalent and/or severe in the baseline scoring than in the final scoring, suggesting that these background injuries largely healed over the course of the study.

In the final welfare indicator scoring, scale losses, snout wounds, and active fin injuries were dominating among fish in both treatment groups, followed by skin bleedings, various eye problems, and thymic bleedings. There was also a significantly higher prevalence and/or severity of snout wounds, various eye problems, and active fin injuries in the final scoring than in the baseline scoring. Most of these injuries are compatible with mechanical injuries that may have been inflicted on the fish during the treatment operations because of dip-netting and confinement in the treatment chamber. The prevalence and/or severity of scale losses, snout wounds, various eye problems, and active fin injuries in the final scoring was, however, significantly higher in the warm water group than in the control group. These injuries were probably due to the behavioural reaction displayed by the fish in the warm water group during the treatments, with vigorous wriggling and violent collisions with the walls of the treatment chamber. On the other hand, it cannot be completely ruled out that the warm water per se inflicted acute or long-term injuries on the fish. Previous studies have, however, not found conclusive evidence that exposure to water at a temperature of 34 °C for 30 s inflicts acute injuries on sedated Atlantic salmon (Kvåle, 2020; Moltumyr et al., 2021).

The findings are largely consistent with previously reported injuries from industrial warm water treatments of salmonids (Gismervik et al., 2018; Poppe et al., 2018; Gismervik et al., 2019b, 2020). However, previously reported bleedings in the brain, nasal pits, palate, gills, and thymuses were not found to be more prevalent after the warm water treatments in this study. The prevalence of macroscopic thymic bleedings was in fact significantly lower in the warm water group in the final scoring than in the baseline scoring, and significantly higher in the control group than in the warm water group in the final scoring. The histopathological examination also revealed no significant differences in histopathological changes in gill, pseudobranch, or thymus between fish in the two treatment groups, but the number of examined fish was admittedly low. One reason for these negative results may be that the time interval of 17–18 days between the second treatment and the final scoring possibly was sufficiently long for potential bleedings to retreat or stop. However, the study of acute injuries by Moltumyr et al. (2021) also showed no significant differences in the prevalence of these bleedings between warm water treated fish and control fish. On the other hand, the fish in the study by Moltumyr et al. (2021) were sedated and held in a soft bag during treatment so that they could not collide with the walls of the treatment cart. In the present study, however, some fish in the warm water group collided so violently with the walls of the treatment chamber that they fainted. In addition to possible concussion, cerebral haemorrhage is therefore a plausible consequence of warm water treatment, especially if the fish have room to reach higher swimming speeds than in this study. Nasal pit, palate, and thymic bleedings are rarely inspected in routine fish health examinations, and the knowledge of their importance as welfare indicators is sparse.

Gismervik et al. (2019a), who studied acute thermal injuries in Atlantic salmon ($\overline{w} = 234 \pm 52$ g) in a pilot laboratory trial, found that exposure to seawater at a temperature of 34–38 °C for 72–140 s caused injuries and/or bleedings in the brain, gills, eyes, and possibly also the nasal cavity and thymuses of the fish. Although salmonids with a mean body weight below 0.5 kg are rarely warm water treated in the aquaculture industry, this finding implies that treatment of Atlantic salmon at equal and somewhat higher water temperatures and longer exposure times than applied in the present study (34 °C for 30 s), poses a serious risk to their health and welfare.

The wound healing rate in teleost fishes depends on various factors,

including stress, environmental conditions, and dietary components (Bullock et al., 1978; Roubal and Bullock, 1988; Jensen et al., 2015). The presence of scale losses, snout wounds, various eye problems, and active fin injuries among fish in both treatment groups in the final scoring in this study, shows that an interval of 17–18 days between the second treatment and the final scoring was too short for the injuries to heal at the prevailing conditions. Thus, repeated treatments at such short time intervals can cause cumulative injuries in the fish.

Injuries in skin, eyes, and fins can activate nociceptors and be painful for fish (Chervova, 1997; Ashley et al., 2007; Roques et al., 2010), while open wounds can lead to osmoregulatory problems (Quilhac and Sire, 1998; Elliott, 2011; Takle et al., 2015) and be a gateway for pathogens (Turnbull et al., 1996; Svendsen and Bøgwald, 1997; Barthel et al., 2003; Noble et al., 2012). Furthermore, skin wounds and scale losses imply damage to the mucus layer that plays a vital role in maintaining fish health by providing a physical and biochemical barrier between the fish and the environment (Shephard, 1994). Eye injuries, eye bleedings, and eye opacity can cause reduced vision and, in the extreme consequence, blindness. This, in turn, can cause the fish to respond more slowly to events in the surroundings and make it difficult for them to forage. Fin injuries have been suggested to reduce fin function and thus the swimming ability and manoeuvrability of fish (Abbott and Dill, 1985; Turnbull et al., 1996; Barthel et al., 2003; Huntingford et al., 2006).

In this study, the fish in the warm water group were treated twice with warm water. In a survey conducted by the Norwegian Veterinary Institute in 2017 (discussed in Gismervik et al., 2018), Norwegian fish health professionals reported that some fish groups in industrial salmon production were treated with various delousing methods more than eight times in the period from the spring delousing to the end of November during their second year in sea. With that many delousing treatments, future studies should emphasise the cumulative load on the fish. The consequences of treating fish with underlying pathologies should also be investigated to enable better risk assessments before treatment and potentially prevent incidents of high mortality. When it comes to warm water treatment specifically, it is important to find the «best practice» that ensures good fish welfare if this delousing method is to continue being used. The results of this study suggest that reducing the fish's behavioural reaction to warm water and/or the impact force in the collisions it causes, may be a mitigating measure.

5. Conclusion

Warm water treatment of Atlantic salmon in a chamber with seawater at a temperature of 34 °C for 30 s twice with an interval of 23–24 days, resulted in a significantly increased prevalence and/or severity of scale losses, snout wounds, various eye problems, and active fin injuries as well as a significantly reduced specific growth rate. The fish displayed an immediate, strong behavioural reaction when exposed to warm water, which was probably the main cause of the detected injuries. Positive welfare effects on Atlantic salmon from getting rid of sea lice were not considered in this study.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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