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Faggruppe farmakologi

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Sluttrapport

SALMON LICE SENSITIVITY TO FRESHWATER AND WARM WATER

Forfattere: Melanie Andrews
Celia Agusti-Ridaura
Tor Einar Horsberg

FINAL REPORT FOR "SALMON LICE SENSITIVITY TO FRESHWATER AND WARM WATER"

DATE	FHF-PROJECT NUMBER	WRITTEN BY
6. oktober 2020	901438	Melanie Andrews Celia Agusti-Ridaura (RNAseq work) Tor Einar Horsberg

PROJECT OVERVIEW

Highlights

- *In vitro* bioassay protocols have been developed to determine sensitivity of salmon lice to freshwater- and warm water bathing treatment
- Copepodid and pre-adult salmon lice tolerated long term exposure to low salinity levels
- Exposure to increased water temperatures, resulted in both copepodid and pre-adult stages being initially affected yet the majority recovered within minutes
- RNAseq studies are completed and are currently analysed
- A literature review and risk assessment were conducted to identify the necessary processes that drive development of tolerance towards freshwater bathing
- The data did not suggest acquired tolerance towards fresh- or warm water in the strains examined
- The variations in the results highlight a need for increased awareness regarding the possibility for resistance to develop over time

Summary

This project aimed to improve understanding of the effects that two commonly used anti-louse treatments, freshwater- and warm water bathing, have on the salmon lice. A literature review and risk assessment were conducted to identify the necessary processes that could drive development of tolerance towards freshwater bathing. In addition, *in vitro* bioassay protocols were developed to allow researchers and fish health workers to test the sensitivity levels in the local lice population. Seven geographically distinct salmon lice populations were included in the development of the *in vitro* bioassay protocols and improving our knowledge of the baseline tolerance to freshwater and warm water treatments. Both the copepodid and pre-adult II stages were initially immobilized by warm water exposure, yet the majority recovered within a few minutes, highlighting the need for filtering effluent water from thermal treatments. Freshwater *in vitro* bioassays suggest that both stages tolerated long term exposure to very low salinity levels, highlighting the importance of lowering the salinity to a level close to zero during treatment. Although both the fresh- and warm water experiments showed some variation in the data, they did not point to acquired tolerance being a problem so far. In addition, RNAseq analyses are currently underway to determine the effect that both freshwater and warm water exposure has on the salmon lice at the molecular level, and to search for potential candidate genes for molecular markers development. Preliminary RNAseq results point to differential regulation of a limited number of genes between groups, however no conclusions can yet be made due to ongoing analysis.

Project participants

<u>Project group</u>

NMBU:	Melanie Andrews, Celia Agusti Ridaura, Tor E. Horsberg
PWSSC:	Maya Groner
UPEI:	Emilie Laurin, Javier Sanchez

<u>Steering group</u>

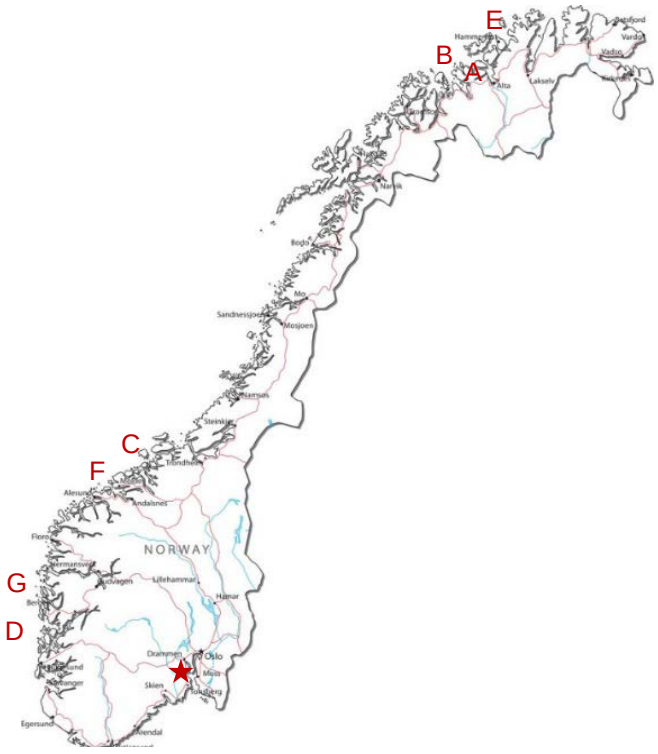
NMBU:	Marit J. Bakke
Marine Harvest:	Gro Vee / Gordon Ritchie
Lerøy:	Bjarne Reinert
Cermaq:	Karl Fredrik Ottem
PatoGen:	Vidar Teis Aspehaug
UPEI / PWSSC:	Crawford Revie
FHF:	Kjell Maroni (observer)

Project background

Work package 1:

This project aimed to develop bioassay methods to increase our understanding of the effect that freshwater bathing and warm water treatments have on the salmon lice. With the primary goal to identify baseline sensitivity levels towards both treatment methods by developing bioassay protocols that can be used in the field to identify possible differences in sensitivity between geographically distant salmon lice populations along the Norwegian coastline. Such knowledge will allow the industry to prioritize treatments in order to improve treatment efficacy as well as gaining a better understanding of how the spread of more resistant lice between regions may have on future treatment routines. Regarding other anti-salmon lice treatments, bioassays are available primarily used to monitor treatment efficacy. However, prior to this project, no such bioassays were available for either freshwater or warm water (thermal) treatments. The project started in August 2018, where we refined our bioassay protocols by testing them on salmon lice populations collected from different regions along the Norwegian coast (Fig. 1). For each salmon lice population, bioassays were conducted following the same procedures, all copepodids and pre-adult II parasites were of similar age and spent the similar amount of time between collection and running the bioassays. The environmental and past treatment data from each collection site and the research facility has been included as additional information.

Fig. 1. Source location for all 7 populations included in the project, star indicates the research facility.



As stated above, development of bioassays is an efficient method to monitor sensitivity and detect resistance. However, bioassays are laborious to conduct and subject to several potential methodological and interpretation errors. Molecular methods have been demonstrated to be a powerful tool for monitoring sensitivity of sea lice to chemicals, since they are a rapid and reliable method for a wide sensitivity screening in field samples (Jansen et al., 2016; Kaur et al., 2016). RNA sequencing (RNAseq) is a potent tool to compare gene expression (as number of transcripts) between selected groups, for all genes simultaneously. This allows for the identification of genes potentially associated with resistance mechanisms and the development of rapid molecular test for sensitivity monitoring. We studied the general gene expression of a louse population under warm water and freshwater treatment conditions, to possibly identify candidate genes for developing molecular markers related to sensitivity loss.

Work package 2:

This work package had two primary goals. The first was to conduct a literature review to evaluate the state of the knowledge on processes necessary for freshwater tolerance to evolve in salmon lice. The second goal was to evaluate the information from the literature review to recommend a risk assessment framework for freshwater tolerance evolution in salmon lice.

RESULTS

Work package 1:

General materials and methods

We conducted research on 7 salmon lice populations collected from northern-, mid-, and south western-Norway, results are provided below. All data were analyzed using probit regression analysis to determine the effective concentration (EC) and upper/lower CI 95. In addition, one-way ANOVA were conducted to determine the effect of treatment on the parasite survival ($\alpha = 0.05$).

The local fish health service collected an average of 15 egg bearing *L. salmonis* during the regular weekly lice count. The salmon lice and egg strings were placed in thermos bottles containing seawater collected on site and were transported via overnight courier to the NMBU Adamstuen laboratory. Upon arrival the eggstrings were removed and placed in hatching containers where they were left to develop until the copepodid stage.

Treatment history for populations included in the study

Egg-bearing adult salmon lice used during this project were collected by fish health personal during routine lice counts. Routine lice counts are conducted so that half of the netpens are checked one week and the other half the next week. This meant that the lice we received were usually sourced from several different netpens, however the treatment data received from the different locations did not specify which pens were treated, merely that a certain treatment was conducted. To avoid including any previously exposed lice, the fish health personal provided us with lice collected before treatments were conducted.

This study included a laboratory strain (pop A) that has been in cultivation for generations without any exposure to treatments. Populations B, F, and G were never exposed to any anti-lice treatments prior to collection. A few netpens in population F were treated with Hydrolicer® in the months prior to collection. Source locations where populations C and D were collected experienced high parasite numbers, with each location conducting freshwater bathing on specific cages prior to collection of the lice for this study. However, we were assured that the lice we received did not originate from netpens that had recently been treated.

Part 1: Freshwater treatment

Materials and Methods

Copepodid L. salmonis bioassays

Eggstrings collected from the field populations were placed in hatching containers as described above, while eggstrings from the cultured population were removed from reservoir adult *L. salmonis* cultured on Atlantic salmon (AquaGen strain). All eggstrings developed undisturbed until the copepodid stage. Once the majority had reached the copepodid stage a portion were used in the copepodid bioassays, whilst the remainder were introduced to new host fish to produce pre-adult II stage lice. Development time from collection of the eggstrings to the copepodid stage takes approximately 2 weeks, depending on the water temperature which had a range of 6.8-13.8°C over the course of this study. Copepodids to be used in the bioassays were placed in a 1 L container and transported to the NMBU Adamstuen campus where they were provided with supplemental air supply and held in a temperature-controlled cabinet (12°C) until commencement of the bioassays, on average 16 hours.

The bioassays were performed in 50 mL containers, each containing an average of 16.6 actively swimming copepodids (range 10 – 27 copepodids). The containers were each assigned to one the following 11 salinities: a seawater control (35 ‰), 23, 21, 19, 17, 15, 13, 11, 9, 7, and 5 ‰ (g/L). A stepwise method of removing seawater and replacing with deionised freshwater was used to gradually achieve the assigned salinity. The exact volume to be removed depends on the salinity of the source seawater, for example the salinity of the seawater used in these bioassays remained consistent at 34.5 ‰. Using an online salinity calculator (Target salinity calculator website) we calculated that in order to reach 23 ‰ we had to remove 15 ml seawater and replace it with 15 ml deionised water, followed by removing 3 ml seawater and adding 3 ml deionised water. The containers were then returned to the temperature-controlled cabinet (12°C) and remained undisturbed for 24 h. Once 24 h had elapsed, each container was examined by emptying its contents into a petri dish and recording the numbers of unaffected and affected copepodids. The status of the copepodids was determined by agitating the water around each copepodid and observing it for signs of normal swimming movement (unaffected); animals exhibiting abnormal movement

or lack of movement were classified as affected. Abnormal movement may be classified as erratic swimming behaviour, an inability to hold position in the water column, and delayed reaction to external stimuli.

Pre-adult II and adult L. salmonis bioassays

Parasites were cultured using two consecutive groups of Atlantic salmon held in a 10 000 L tank, these included an initial 10 fish (ranging 3-4.5 kg) followed by 50 fish (ranging 200-300 g). The water level was reduced until it was a few centimetres above the dorsal fin, the fish were then corralled together using a gate-like barrier. The copepodids were introduced into the area with the corralled fish and left undisturbed for 2 hours, after which the barrier was removed, and water flow resumed. The fish were left undisturbed until the majority had reached the pre-adult II stage, they were corralled together and transferred individually into an anaesthetic bath (metacaine, 100 mg/L). Once sufficiently anaesthetized (2 - 3 minutes), each fish was examined, and all parasites were removed using forceps and placed in 1L containers holding seawater. The parasites were then transported to the laboratory where they were provided with an air supply and left in a temperature-controlled cabinet (12°C) until commencement of the bioassays, on average 16 hours.

Bioassays were performed in 1 L beakers containing 500 mL seawater and 10 PA II L. salmonis. Each beaker was randomly designated one of the following salinities: seawater control (35 ‰), 20, 15, 10, 5 and 0 ‰. As described in the previous section, a stepwise method of removing seawater and replacing it with deionised freshwater was used to gradually achieve the assigned salinity. The containers were then returned to the temperature-controlled cabinet (12°C), provided aeration, and left undisturbed for 24 h. After the 24 h had elapsed, the containers were vigorously stirred, allowed to settle, and stirred again before being emptied over a sieve. If a salmon louse remained attached to the wall of the container or exhibited normal swimming behaviour, it was considered unaffected; those parasites that did not attach were placed in a petri dish and observed. If they were unable to swim or attach, they were considered affected.

Results

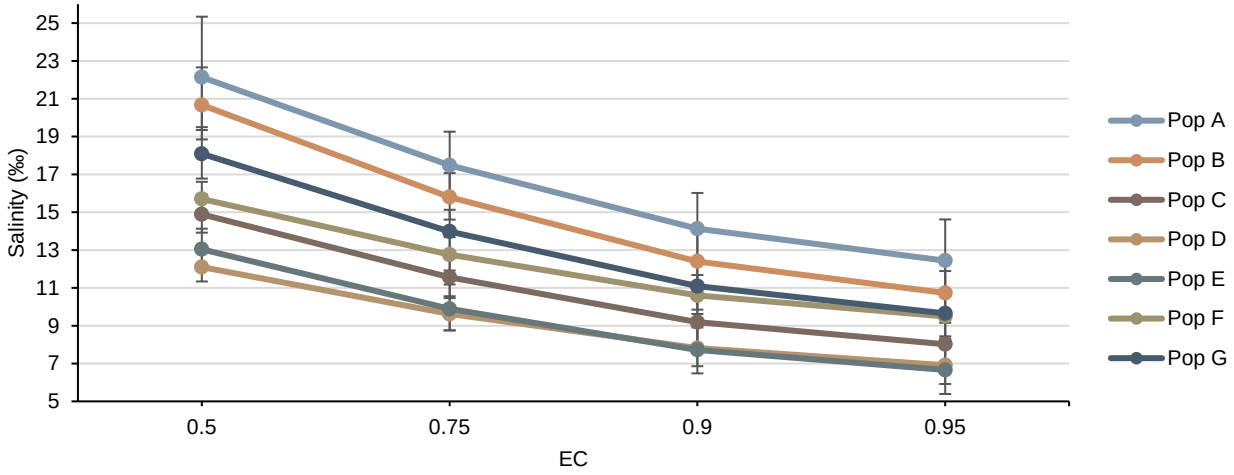
Exposure of copepodids to reduced salinity

Copepodids from 7 salmon lice populations were used in 24h salinity exposure bioassays, with the aim to determine whether copepodid survival rate decreases over an extended exposure time and whether there were differences between populations (Table 1, Fig. 2). Pop A and B exhibited lower tolerance to decreased salinity, they followed similar trends in response to decreased salinity with all copepodids being affected by 9 ‰ (Table 1). In comparison, pop D and pop E had a higher percentage of unaffected copepodids at 9 ‰ with 31,6% and 31,5% respectively (Table 1), the remaining populations had low a percentage of unaffected copepodids at this salinity. At 7 ‰, pop D had 23,7%, followed by pop E (10,7%), and finally pop C at 2,9%. A curve has been provided illustrating the effective concentration (EC) at different levels for all populations; EC_{50}, EC_{75}, EC_{90} and EC_{95} (Fig. 2). Pop D and pop E displayed higher tolerance with an EC_{50} value of 12.1 ‰ and 13 ‰ respectively, compared to population A (22,1 ‰) and pop B (20,7 ‰) ($F_{(1,21)} = 48,7$, $p = 0,0001$).

Table 1. Percentage (±SE) unaffected copepodids following 24h exposure to reduced salinity

Salinity (‰)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
35	100±0	100±0	100±0	100±0	100±0	100±0	100±0
23	31,3±5,5	46,4±46,4	100±0	97,4±2,6	93,3±0	94,1±5,9	77,5±2,5
21	41,2±5	43,8±36,1	50,2±43,9	94,7±5,3	82,9±7,9	79,4±7,2	53,3±0
19	45±5,5	52,6±36,1	83,3±16,7	90,4±3,7	77,3±3,9	72,8±2,2	56,3±2,5
17	18,2±9	10±10	69,7±0,9	84,2±0	76,1±9,5	56,1±16,1	36,1±13,9
15	14,3±9	30±30	53,3±13,3	65,8±4,7	57,1±28,6	50±0	32,9±10,8
13	0±0	52,8±23,1	83,3±10,7	90,4±6,1	77,3±21,4	72,8±3,3	56,3±7,9
11	9,5±9,5	11,5±11,5	21,3±3,7	33,6±11,4	34,6±27,9	16,8±4,3	10,2±3,1
9	0±0	0±0	10,6±4,8	31,6±15,8	31,5±10,1	6,3±6,3	5,9±5,9
7	0±0	0±0	2,9±0	23,7±0	10,7±0	0±0	5,9±0
5	0±0	0±0	0±0	0±0	0±0	0±0	0±0

Fig. 2. Curve illustrating the percentage of immobilised copepodids as the salinity decreases (including upper and lower CI 95)



Exposure of pre-adult II to reduced salinity

Twenty-four hour salinity exposure bioassays were conducted on the pre-adult II stage for each of the 7 salmon lice populations, with the aim to determine whether survival rate decreases over an extended exposure time and to determine whether there were differences in survival between populations (Tables 2-4, Fig 3). All populations maintained relatively high percentage of unaffected lice (over 50%) up until 5 ‰ (Table 2), with very little differences between females (Table 3) and males (Table 4). A curve for each population illustrating the effective concentration (EC) at different levels; EC_{50}, EC_{75}, EC_{90} and EC_{95} has been provided in Fig. 3. The most sensitive were pop C (5,4 ‰) and pop D (4,8 ‰), while the remaining populations had very similar EC_{50} values ranging between 3,2 - 4 ‰, while pop B exhibited the highest tolerance with an EC_{50} value of 2.1 ‰ ($F_{(1,5)}$ = 27,5, p = 0,0063).

Table 2. Percentage (%) unaffected pre-adult II following 24h exposure to reduced salinity

Salinity (‰)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
35	100	100	100	100	100	100	100
20	90	100	100	57,5	100	100	100
15	100	100	80	90	90	91,7	61,7
10	100	60	40	100	80	90	87,5
5	70	90	80	60	80	90	100
0	0	30	0	0	0	0	0

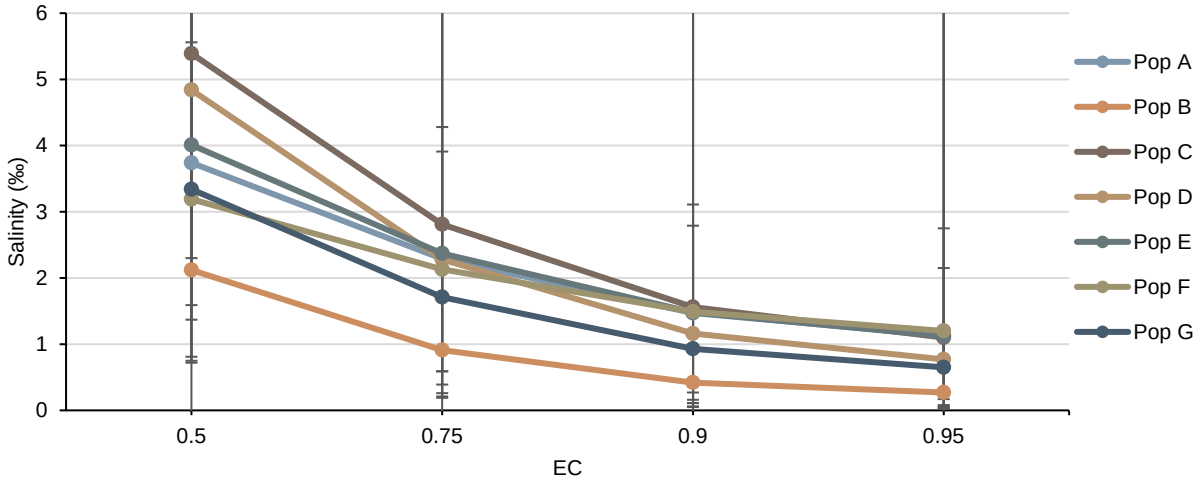
Table 3. Percentage (%) unaffected pre-adult II females following 24h exposure to reduced salinity

Salinity (‰)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
35	100	100	100	100	100	100	100
20	80	100	100	40	100	100	100
15	100	100	80	100	100	83,3	83,3
10	100	60	80	100	100	80	100
5	80	100	80	40	80	80	100
0	0	20	0	0	0	0	0

Table 4. Percentage (%) unaffected pre-adult II males following 24h exposure to reduced salinity

Salinity (‰)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
35	100	100	100	100	100	100	100
20	100	100	100	75	100	100	100
15	100	100	80	80	80	100	40
10	100	60	0	100	60	100	75
5	60	80	80	80	80	100	100
0	0	40	0	0	0	0	0

Fig. 3. Curve illustrating the percentage of immobilised pre-adult II salmon lice following 24h exposure to reduced salinity (including upper and lower CI 95)



Comparing the results with source salinity

Populations included in this study originated from northern-, mid-, and south-western Norway and they were collected during both the warm and cold seasons, these differences were planned in order to determine whether different environmental conditions may influence susceptibility to freshwater bathing. The differences in average salinity were not very high with the research facility having an average of 33,5 ‰, whilst pop E had the lowest (28,3 ‰) and pop G the highest (33,5 ‰). Pop A is considered the control lab population as it has been in culture for many generations and is known to be sensitive to numerous treatment compounds. It originated in an area near pop B (Fig. 1), however it seems that even though it has been in culture for many years it reflects the trend shown in pop B when regarding the copepodids (Fig. 2), however the pre-adults seem to be less sensitive (Fig. 3). Regarding pop E which experienced an average salinity of 29 ‰, resulted in one of the highest survival rates after long-term exposure to low salinity for the copepodid stage (Fig. 2), however the pre-adult II stage proved to be more sensitive (Fig. 3). The aim of this project was to create a protocol that could determine baseline sensitivity to low salinity levels. Currently it is not possible to conclude whether salinity levels at the site of origin determines sensitivity to lower salinities, to answer that question more populations would have to be sampled and more in-depth analysis of the environmental factors should be conducted. However, the results do indicate that freshwater bathing can vary in efficacy between the different regions.

Effect of long-term exposure to lower salinity: copepodid vs the pre-adult II

The responses between the two developmental stages have been provided in Table 5. The EC_{50} salinity data for the pre-adult II stage had a very narrow range from as low as 2,1 ‰ (pop B) to a high of 5,4 ‰ (pop C). The copepodid stage displayed a wider range with a low of 12,1 ‰ (pop D) to a high of 22,1 ‰ (pop A) (Table 5). There seems to be no connection in tolerance levels between the two developmental stages, including a greater number of sites may address this however it does not fall into the scope of the current project.

Table 5. Comparing the effective concentrations (EC) between the copepodid and pre-adult II stages for 7 salmon lice populations after 24 h exposure to reduced salinity. Including 95% CI.

Location	Copepodid			Pre-adult II		
	EC_{50}	Lower 0,95	Upper 0,95	EC_{50}	Lower 0,95	Upper 0,95
Pop A	22,1	2,8	3,2	3,7	2,4	6,5
Pop B	20,7	1,8	2,0	2,1	1,3	3,4
Pop C	14,9	1,0	1,1	5,4	3,1	7,2
Pop D	12,1	0,8	0,8	4,8	3,3	9,9
Pop E	13,0	1,0	1,1	4,0	2,7	7,8
Pop F	15,7	0,9	0,9	3,2	2,5	11,0
Pop G	18,1	1,3	1,4	3,3	2,6	11,6

A manuscript from the studies has been published in Aquaculture.

Part 2: Warm water treatment

Materials and methods

Copepodid L. salmonis bioassays

Eggstrings collected from the field populations were placed in hatching containers as described above, while eggstrings from the cultured population were removed from reservoir adult *L. salmonis* cultured on Atlantic salmon (AquaGen strain). All eggstrings developed undisturbed until the copepodid stage. Once the majority had reached the copepodid stage a portion were used in the copepodid bioassays, whilst the remainder were introduced to new host fish to produce pre-adult II stage lice. Development time from collection of the eggstrings to the copepodid stage takes approximately 2 weeks, depending on the water temperature which had a range of 6.8-13.8°C over the course of this study. Copepodids to be used in the bioassays were placed in a 1 L container and transported to the NMBU Adamstuen campus where they were provided with supplemental air supply and held in a temperature-controlled cabinet (12°C) until commencement of the bioassays, on average 16 hours.

Producers of the Thermolicer™ calculates treatment temperatures as Δ°T, which is the difference between the start (maintenance) temperature and treatment temperature allowing the comparison between different geographic regions. However, start temperatures used in this study were generally more stable between experiments and Δ°T was not included. Eleven temperatures (control at 12°C, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40°C) were included in these bioassays. Each replicate was assigned a 50 ml container, with a base of 100 µm gauze, placed in a container holding seawater at 12°C. An average of 15 healthy copepodids were pipetted into each replicate. Glass bottles containing 200 ml seawater were placed in water baths and allowed to reach the desired temperature. Once the water had reached the required temperature, the copepodids were exposed to the heated seawater for 2 min, the copepodids were then immediately returned to the holding container at 12°C. The number of unaffected/affected copepodids was recorded, after which the copepodids were returned to the temperature-controlled cabinet and left undisturbed for 24h. After 24h the number of unaffected/affected copepodids was recorded. Copepodids were determined to be affected if they were immobile or exhibited abnormal swimming behaviour when disturbed, whereas copepodids were classified as unaffected if they exhibited normal swimming and avoidance behaviour when disturbed.

Pre-adult II L. salmonis bioassays

Parasites were cultured using two consecutive groups of Atlantic salmon held in a 10 000 L tank, these included an initial 10 fish (ranging 3-4.5 kg) followed by 50 fish (ranging 200-300 g). The water level was reduced until it was a few centimetres above the dorsal fin, the fish were then corralled together using a gate-like barrier. The copepodids were introduced into the area with the corralled fish and left undisturbed for 2 hours, after which the barrier was removed, and water flow resumed. The fish were left undisturbed until the majority had reached the pre-adult II stage, they were corralled together and transferred individually into an anaesthetic bath (metacaine, 100 mg/L). Once sufficiently anaesthetized (2 - 3 minutes), each fish was examined, and all parasites were removed using forceps and placed in 1L containers holding seawater. The parasites were then transported to the laboratory where they were provided with an air supply and left in a temperature-controlled cabinet (12°C) until commencement of the bioassays, on average 16 hours.

Seven temperatures (control at 12°C, 30, 31, 32, 33, 34, 35°C) were included in these bioassays. One replicate per treatment temperature was assigned to a 500 ml flask of seawater, 5 healthy male and 5 healthy female pre-adult II lice were transferred into each flask. The replicate was stirred allowing the parasites to attach to the flask, it was then drained over a sieve, to catch any detached lice. Heated seawater at the desired temperature was poured into the flask, allowed to stand for 2 min, after which it was stirred and emptied over a sieve. The number of unaffected/affected parasites was recorded and then returned, using forceps, to the original flask containing 12°C seawater. The flasks holding the treated parasites were returned to the temperature-controlled cabinet, provided additional aeration and allowed to stand undisturbed for 24 h. After 24 h, the number of unaffected/affected parasites was recorded. Parasites were determined to be affected if they were immobile, unable to attach and remain attached to the flask, or exhibited abnormal swimming behaviour when disturbed. Parasites were classified as unaffected if they could firmly attach to the flask, or if they were able to avoid being disturbed by swimming or moving away from the disturbance and reattaching to the flask.

Results

Exposure of copepodids to increased water temperature

Exposure bioassays were conducted using copepodids from 7 salmon lice populations where the copepodids were exposed to increased water temperature for 2 min. The number of unaffected copepodids were noted immediately following exposure

(Table 6), they were then returned to 12°C water and the number of unaffected copepodids were counted 24h after exposure (Table 7). At the initial 2 min count a gradual decrease in the percentage of unaffected copepodids was observed in all populations. Pop B (46,6 %) had fewer unaffected copepodids at 31°C, however none were observed at 32°C. In comparison unaffected copepodids were observed at 33°C in pop A (20%), pop C (28,6%), and pop F (21,1%). Pop C was the only population with unaffected copepodids at temperatures above 33°C, with 2,9% active at 38°C (Table 6).

Twenty-four hours following exposure, the majority of copepodids recovered in all populations at temperatures up to 34°C (Table 7). At 34°C pop C had the most unaffected at 86,9%, followed closely by pop B (82,9%). Pop F and pop G had the lowest at 55,2% and 55% respectively. At 36°C, all copepodids were affected in pop A, whereas the percentage of unaffected copepodids in pop C remained high at 74,1%. At 38°C unaffected copepodids were observed in pop C, D, F and G, whilst pop B had unaffected copepodids at 39°C. These temperatures are much higher than what would be used on site with 34°C being the max recommended temperature.

Table 6. Percentage (±SE) unaffected copepodids directly after 2 min exposure to increased temperatures

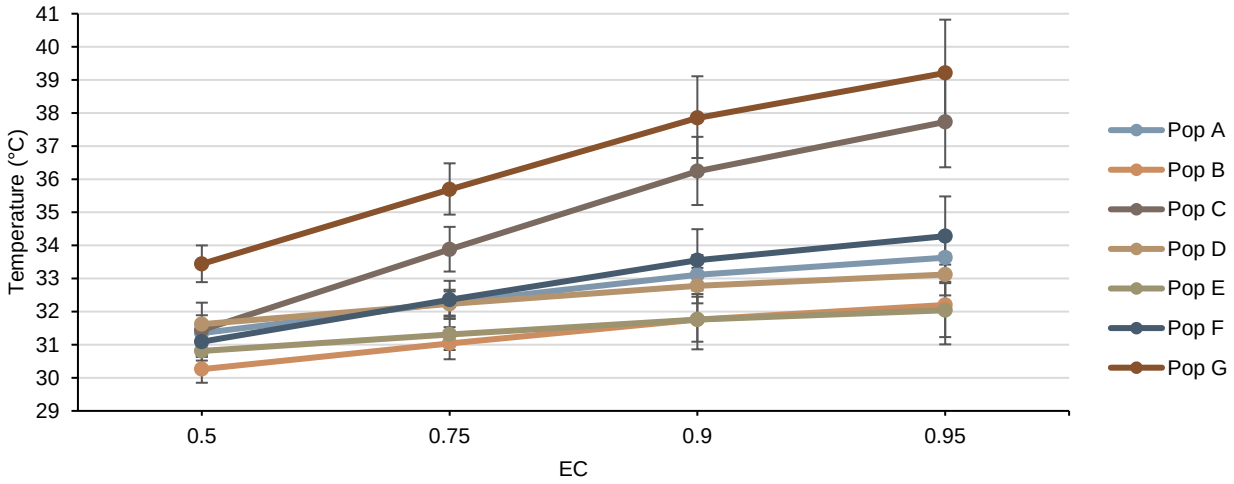
Temperature (°C)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
12	100±0	100±0	100±0	100±0	100±0	100±0	100±0
30	84,9±2,6	46,4±0,3	59,4±15,6	94,1±5,9	80,1±3,2	42,6±7,4	84,6±7,7
31	59,4±3,8	46,6±0,5	67,4±5,9	72,1±7,9	55,4±13,8	90,8±2,5	75±0
32	29,2±4,2	0±0	51,7±1,7	50,2±2,8	0±0	31,9±6,9	58,3±8,3
33	20,0±13,3	0±0	28,6±21,4	0±0	0±0	21,1±2,4	0±0
34	0±0	0±0	0±0	0±0	0±0	0±0	0±0
35	0±0	0±0	26,3±13,8	0±0	0±0	0±0	0±0
36	0±0	0±0	17±4,5	0±0	0±0	0±0	0±0
37	0±0	0±0	10,4±10,4	0±0	0±0	0±0	0±0
38	0±0	0±0	2,9±2,9	0±0	0±0	0±0	0±0
39	0±0	0±0	0±0	0±0	0±0	0±0	0±0
40	0±0	0±0	0±0	0±0	0±0	0±0	0±0

Table 7. Percentage (±SE) unaffected copepodids 24h after 2 min exposure to increased temperatures

Temperature (°C)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
12	100±0	100±0	100±0	100±0	100±0	100±0	100±0
30	90,6±9,4	96,7±3,3	100±0	91,5±3,3	100±0	84,9±2,6	84,6±0
31	73,2±10,1	77,6±7,0	100±0	93,1±0,2	87,8±4,5	84,1±4,1	68,8±6,3
32	76,4±1,4	100±0	95,8±4,2	88,9±0,6	90±10	76,4±1,4	65±15
33	60,0±0	52,2±11,0	89,7±4	89,7±4,7	78,3±5	66,7±2	53,1±3,1
34	59,6±6,7	82,9±2,9	86,9±6,9	87,6±1,9	67,1±2,9	55,2±11,5	55±5
35	18,8±3,4	10,1±4,2	96,7±3,3	76,4±1,4	71,7±1,7	51,1±13,6	49,5±14,2
36	0±0	19,9±7,4	74,1±11,6	27,2±3,2	19,6±5,4	27,8±5,6	29±0,4
37	0±0	3,3±3,3	66,3±24,6	20,1±3,4	0±0	36,7±10,4	12,5±12,5
38	0±0	0±0	16,3±1,3	10,1±4,2	0±0	3,1±3,1	6,7±6,7
39	0±0	7,1±7,1	0±0	0±0	0±0	0±0	0±0
40	0±0	0±0	0±0	0±0	0±0	0±0	0±0

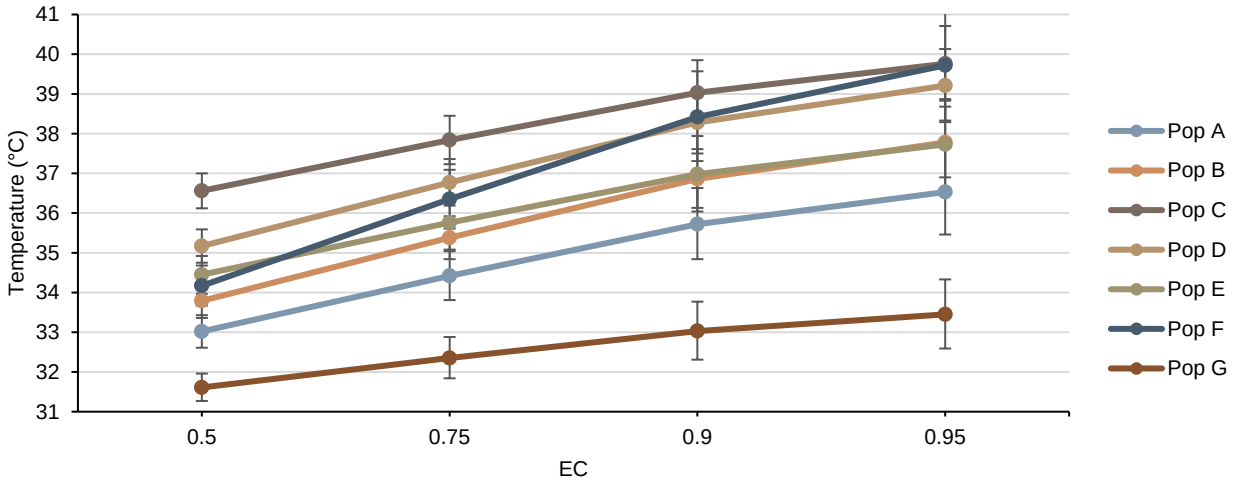
A curve for each population illustrating the effective concentration (EC) at different levels; EC_{50}, EC_{75}, EC_{90} and EC_{95} immediately following 2 min exposure to increased water temperatures has been provided (Fig. 4). The EC_{50} results are very similar for most populations, with the lowest being 30,3°C however pop G indicated a significantly higher tolerance with an EC_{50} of 33,4°C ($F_{(1,23)}$ = 32,5, p = 0,0001).

Fig. 4. Curve illustrating the percentage of immobilised copepodids immediately following 2 min exposure to increased water temperatures (including upper and lower CI 95)



A curve for each population illustrating the effective concentration (EC) at different levels; EC_{50}, EC_{75}, EC_{90} and EC_{95} population 24h following 2 min exposure to increased water temperatures is provided in Fig. 5. The EC_{50} values have spread out compared to those from the 2 min observations. Pop C showed a significantly greater tolerance with an EC_{50} value of 36,6°C ($F_{(1,23)}$ = 5,8, p = 0,024), whereas pop G was the least tolerant with 31,6°C ($F_{(1,23)}$ = 18,1, p = 0,0003).

Fig. 5. Curve illustrating the percentage of immobilised copepodids 24h after 2 min exposure to increased water temperatures (including upper and lower CI 95)



Exposure of the pre-adult II stage to increased water temperature

Exposure bioassays were conducted on the pre-adult II stage from 7 populations where the lice were exposed to increased water temperatures for 2 min. Immediately following 2 min exposure the percentage of affected/unaffected lice were recorded (Table 8). They were then returned to a temperature-controlled cabinet (12°C) and left for 24h after which the percentage of affected/unaffected lice were recorded (Table 9). At the 2 min check, pop B showed greater tolerance with 100% active (31°C) and 63% active (32°C) immediately following exposure. Closely followed by pop C and G had 30% and pop F had 0% at 30°C (Table 8). In comparison, all lice in pop E were affected at 30°C however 10% were then unaffected at 35°C (Table 8). Activity counts 24h after exposure showed that until 35°C all populations had relatively high percentage of unaffected parasites (Table 9). Pop A with the highest 83,3% and pop F with 33%. Illustrating that pre-adult II stage lice exhibit an initial shock response from which the majority recover relatively rapidly.

Table 8. Percentage unaffected pre-adult II immediately after 2 min exposure to increased water temperatures

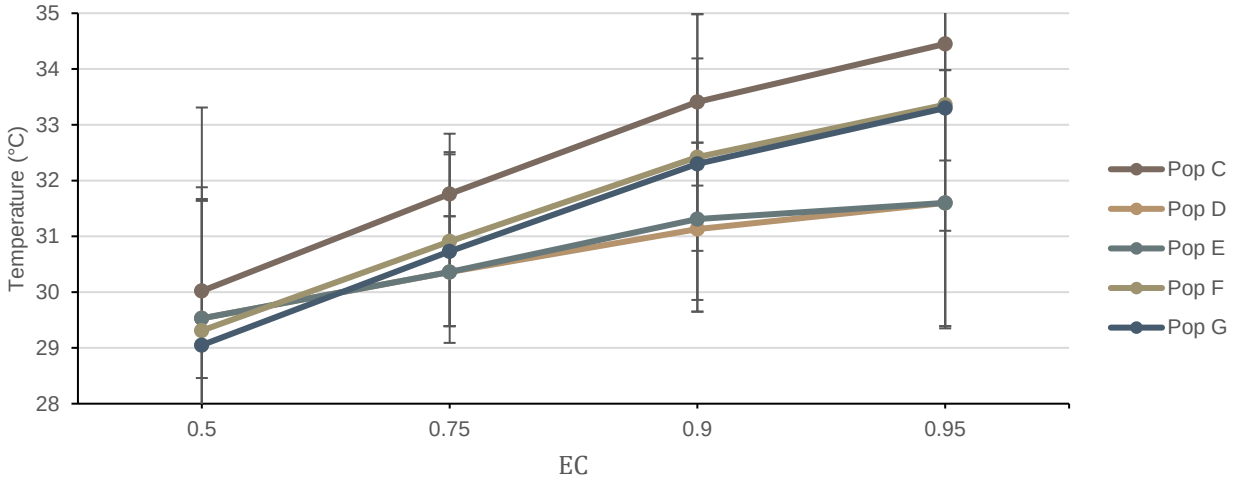
Temperature (°C)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
12	100	100	100	100	100	100	100
30	100	100	60	30	0	32,5	20
31	0	100	20	20	0	37,5	30
32	0	63,3	30	0	0	0	30
33	0	0	0	0	0	0	0
34	0	0	20	0	0	10	0
35	0	0	0	0	10	0	0

Table 9. Percentage unaffected pre-adult II 24h after 2 min exposure to increased water temperatures

Temperature (°C)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
12	100	100	100	100	100	100	100
30	83,3	83,3	83	100	50	50	100
31	100	87,5	67	67	50	50	100
32	100	87,5	100	67	25	33	50
33	100	33,3	83	100	75	50	67
34	100	29,1	50	100	17	83	67
35	83,3	50	50	67	67	33	67

A curve for each population illustrating the effective concentration (EC) at different levels; EC_{50}, EC_{75}, EC_{90} and EC_{95} population 24h following 2 min exposure to increased water temperatures is provided in Fig. 6. Pop C (30°C) had a slightly higher tolerance than the other populations which were grouped between 29-29,5°C ($F_{(1,6)} = 16,9$, $p = 0,0092$).

Fig. 6. Curve illustrating the percentage of immobilised pre-adult II immediately following 2 min exposure to increased water temperatures (including upper and lower CI 95)



When pooling the groups to compare the sexes, little difference was seen at either the 2 min count (Tables 10 and 11) or at the 24h count (Tables 12 and 13). In the case of the pre-adult II stage, it was not possible to include dose-response curves

as no temperatures resulted in 100% mortality. Personal observations during the bioassays indicate that the parasites recover within minutes following warm water treatment.

Table 10. Percentage unaffected pre-adult II females immediately after 2 min exposure to increased temperatures

Temperature (°C)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
12	100	100	100	100	100	100	100
30	100	100	40	20	0	40	40
31	0	100	0	0	0	50	20
32	0	60	40	0	0	0	20
33	0	0	0	0	0	0	0
34	0	0	20	0	0	20	0
35	0	0	0	0	20	0	0

Table 11. Percentage unaffected pre-adult II males immediately after 2 min exposure to increased temperatures

Temperature (°C)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
12	100	100	100	100	100	100	100
30	100	100	80	40	0	25	0
31	0	100	40	40	0	25	40
32	0	67	20	0	0	0	40
33	0	0	0	0	0	0	0
34	0	0	20	0	0	0	0
35	0	0	0	0	0	0	0

Table 12. Percentage unaffected pre-adult II females 24h after 2 min exposure to increased water temperatures

Temperature (°C)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
12	100	100	100	100	100	100	100
30	100	100	67	100	67	67	100
31	100	75	67	67	100	100	100
32	100	100	100	67	0	67	67
33	100	33	100	100	100	100	67
34	100	33	33	100	33	67	100
35	67	33	67	67	67	67	67

Table 13. Percentage unaffected pre-adult II males 24h after 2 min exposure to increased water temperatures

Temperature (°C)	Pop A	Pop B	Pop C	Pop D	Pop E	Pop F	Pop G
12	100	100	100	100	100	100	100
30	67	67	100	100	33	33	100
31	100	100	67	67	0	0	100
32	100	75	100	67	50	0	33
33	100	33	67	100	50	0	67
34	100	25	67	100	0	100	33
35	100	67	33	67	67	0	67

Comparing the EC_{50} values for the copepodid and pre-adult II stages we can see that the copepodid stage were slightly more tolerant to increased temperatures than the pre-adult II lice, apart from pop B which had 30,3°C for the copepodids and 32°C for the pre-adult II lice (Table 14), however there was no significant difference in between the stages ($F_{(1,6)} = 3,2$, $p = 0,134$). Pop E was the only population where the EC_{50} values were similar for both life stages.

Table 14. Comparing the EC_{50} values between the copepodid and pre-adult II stages for 7 salmon lice populations immediately after 2 min exposure to increased water temperature. Including 95% CI.

Location	Copepodid			Pre-adult II		
	EC_{50}	Lower 0,95	Upper 0,95	EC_{50}	Lower 0,95	Upper 0,95
Pop A	31,4	0,3	0,3	30,0	0,0	0,0
Pop B	30,3	0,4	0,4	32,0	0,0	0,1
Pop C	31,4	0,8	0,8	30,0	1,5	1,7
Pop D	31,6	0,3	0,3	29,5	2,0	2,1
Pop E	30,8	0,3	0,3	29,5	2,0	2,1
Pop F	31,1	0,4	0,4	29,3	2,4	2,6
Pop G	33,4	0,5	0,6	29,1	3,8	4,3

Comparing the results with source water temperatures

The average monthly seawater temperature ranged from pop E having the lowest at 5,8°C (2,4-9,8°C) to pop G with the highest of 10,7°C (3,1-16,4°C), while the research facility was 9,3°C (6,9-12,7°C). Pop E were in general less tolerant to increased water temperatures than the other populations (Table 14). This population comes from an area in northern Norway where the recorded average monthly seawater temperatures never went above 10°C, in addition they were sampled from the field during the winter. However pop G was sampled at higher temperatures and resulted in similar results. In comparison, pop B also originates from northern Norway but was sampled during the summer with an average monthly temperature above 10°C. Here the copepodids were relatively sensitive, however the pre-adult II stage was more tolerant (Table 15). The inconsistency of these results indicates that sensitivity to warm water treatment is highly variable. An increased number of populations should be studied in both the winter and summer months to determine whether there is a connection, however this was not included in the scope of the current project.

A manuscript from the studies has been accepted for publication in Aquaculture.

Part 3: RNAseq studies: warm and freshwater treatments

Materials and methods

Salmon louse strain

The louse population chosen for the study was collected from Oksen (Pop. G) which is situated in the Øygarden zone, north of Bergen. The area historically was treated with azimethiphos, emamectin benzoate, freshwater bathing and warm water bathing. However, the lice were collected from fish that had never received any treatment since their introduction to the sea cages. Those characteristics maximize the probability of obtaining a louse population with a broad range of sensitivities to warm and freshwater treatments, essential requirement for obtaining enough sensitive and resistant lice for the RNAseq study.

Bioassays and RNAseq samples

L. salmonis eggstrings were collected from the Oksen (Pop. G) population allowing for the infection of two 10,000L tanks each holding approximately 50 Atlantic salmon. One tank was used to conduct preliminary freshwater and warm water bioassays to determine the EC_{50} value for both treatments. The second tank was used for selecting sensitive and resistant lice to warm and freshwater for the RNAseq study. This selection was performed using a single discriminating dose bioassay based on the EC_{50} value obtained in the preliminary bioassays: 29.5°C for warm water and 3.3 ‰ for freshwater. Adult males and females, most females carrying the first set of egg strings, were used, and bioassays were run the day after lice were collected from fish.

For the warm water selection bioassay for RNAseq, salmon lice were collected a few days after the preliminary bioassays from the second 10,000L tank. Freshwater RNAseq bioassay was run on the second louse generation, cultured from eggs collected prior to conducting the warm water assay. The egg strings were collected, allowed to hatch and develop to the infective copepodid stage. The copepodids (approximately 40 copepodids/fish) were introduced to a 10,000L tank holding approximately 50 Atlantic salmon and allowed to develop until the majority had reached the adult stage. The fish were anaesthetized (metacaine, 100 mg/L) and all lice were removed from the fish and placed in 1L containers holding seawater. They were then transported to the laboratory where they were provided aeration and placed in a temperature-controlled cabinet at 12°C until the bioassays were conducted.

For the warm water bioassay, lice were exposed to 30°C for 2 min and were left afterwards at 12°C for 24h with constant aeration. Two sets of samples were collected: one set immediately after the 30°C exposure for 2 min (T2 samples) and another set after 24h at 12°C (T24 samples). A bioassay control group, kept at 12°C with constant aeration, were also included. For the freshwater bioassay, lice were exposed to 2 ‰ salinity for 24 h at 12°C with constant aeration. We used 2 ‰ instead of 3.3 ‰ because the 3.3 ‰ bioassay did not yield any affected lice. Two control groups were included: the bioassay control group (kept at 33‰ salinity) and lice fixed the same day of collection from fish. Immobilization rate was recorded immediately after the exposure: Parasites affected after the exposure were considered sensitive, whereas parasites that were not visibly affected were considered resistant. Lice were classified as affected when they were completely immobilized at the bottom of the container, and unaffected when they were able to attach to the container wall and swim actively. Lice were fixed in RNAlater immediately after the exposure and kept at −80 °C following ~24h at room-temperature. Table 15 shows the samples obtained in the bioassays and included in the RNAseq study. Only adult females were used.

Table 15. Data on the 40 samples enrolled in the warm and freshwater RNAseq studies. N: sample size. All adult female lice.

Treatment	Group	N	Description
Warm water	Control	3	Lice used as control bioassay group, kept at 12°C.
	T2-S	5	Sensitive lice exposed to 30°C for 2 min.
	T2-R	5	Resistant lice exposed to 30°C for 2 min.
	T24-S	6	Sensitive lice exposed to 30°C for 2 min and left at 12°C for 24h afterwards
	T24-R	5	Resistant lice exposed to 30°C for 2 min and left at 12°C for 24h afterwards

Freshwater	Control	3	Lice fixed the same day of collection from fish.
	Control-B	3	Lice used as control bioassay group, kept at 33 ‰ salinity.
	S	5	Sensitive lice exposed to 2 ‰ salinity for 24 h at 12°C
	R	5	Resistant lice exposed to 2 ‰ salinity for 24 h at 12°C

RNAseq: Transcriptome analysis

RNA extraction

Total RNA was extracted from the 40 individual adult females using a Trizol protocol combined with RNeasy Mini kit for animal tissues (Qiagen, CA, USA) (one individual per extraction). Lice tissues were disrupted and homogenized in 1 ml Trizol using TissueLyser MM 301 (Qiagen Retsch) and one stainless steel bead of 5 mm diameter (Qiagen). After mixing with 0.2 ml of chloroform and a centrifugation step, the aqueous phase was transferred to a new vial and mixed with one volume of 70% ethanol. Total RNA was then isolated with RNeasy spin columns following manufacturer's protocol. Genomic DNA was removed from the extracted RNA (10 µg) with Turbo DNA-free TM kit (TURBO™ DNase Treatment and Removal Reagents, Ambion, Life Technologies Thermo Fisher Scientific, USA). Subsequently, the RNA was cleaned and concentrated with RNA Clean & ConcentratorTM-5 (Zymo Research). The RNA was quantified with Epoch™ Multi-Volume Spectrophotometer System (BioTek Instruments, Inc. VT, USA) and the quality was checked with a 2100 Bioanalyzer instrument (Agilent Technologies) and the Agilent RNA 6000 Nano kit.

RNAseq analysis

Total RNA samples were used for library preparation and Illumina sequencing at the Norwegian Sequencing Centre (Oslo, Norway). Forty RNAseq libraries (one per individual lice), each with unique index barcodes, were prepared using the TruSeq Stranded total RNA library preparation Kit v2 (Illumina, USA) by following manufacturer's protocol including the polyA enrichment step. Libraries were pooled together and sequenced on NovaSeq 6000 platform (Illumina, USA) using 150 bp paired end High output reagents. Raw .bcl files were generated using RTA v2.4.11 and were later demultiplexed (using the sample specific index) and converted to fastq format using bcl2fastq v2.17.1.14.

Gene expression analysis

Demultiplexed raw reads were cleaned using Trimmomatic v0.33 (Bolger et al., 2014) to remove/trim low quality reads and sequencing adapters as well as using BBMap v34.56 (https://sourceforge.net/projects/bbmap/) to remove reads mapping to PhiX genome (Illumina spike-in). Cleaned fastq reads for each parasite were aligned to the *L. salmonis* transcriptome (coding sequences) using Hisat2 v2.1.0 (Kim et al., 2015). The transcriptome file from ENSEMBL release 44 (ftp://ftp.ensemblgenomes.org/pub/metazoa/release-44/fasta/lepeophtheirus_salmonis) contained the predicted transcriptome from genomic data. Unmapped reads were filtered out using Samtools version 1.4 (Li et al., 2009). Gene annotation files in GTF format were generated for each parasite and then merged using Cufflinks version 2.2.1. (Trapnell et al., 2010). Counts of fragments aligning to each transcript were calculated using featureCounts version 1.5.2. (Liao et al., 2014). Analysis of the differential expression within each group was done using DESeq2 (Love et al., 2014) (default settings for the count normalization method). The significance level was set to $\alpha = 0.05$.

Results and discussion

RNAseq expression analysis: Warm water

RNAseq gene expression analysis (DESeq2) for the warm water experiment showed that the T2-R group had only eight genes differentially regulated compared to the corresponding sensitive group T2-S: five down-regulated and three up-regulated (Fig. 7 and Table 16). The putative annotation of those genes is shown in Table 2. There was not any gene differently regulated between T24-S and T24-R groups. The control group had 102 genes differentially regulated compared to the T2-S group, and 370 genes compared to T2-R group. In addition, the control group had very few (less than 10) genes differentially regulated compared to the T24 groups.

These results may indicate that only 2 min exposure to warm water is able to change the expression of several genes in salmon lice. However, after 24 h in normal conditions (12ºC), the gene expression is very similar to the unexposed control group, and no differences can be found between sensitive and resistant T24 lice. Differential regulation of gene expression has been suggested in sea lice after 20-30 min of hydrogen peroxide exposure (Valenzuela-Muñoz et al, 2020, Agustí-Ridaura unpublished study in *L. salmonis*), and it has been demonstrated after 2 h of hydrogen peroxide injection in a penaeid shrimp (Wang et al., 2012).

The gene expression differences found between T2-S and T2-R could point to an incipient selection of less sensitive lice to warm water and might be used to develop potential molecular markers for resistance. Nevertheless, it is important to stress that those differences were found after the exposure of the lice to warm water. The study of resistant individuals not exposed to warm water may be important to validate the findings. A large interindividual variation was found in each louse group for some genes, being difficult to detect statistical significance in the analysis. This variation could be due to a low selection pressure of the strain and/or the short exposure time (2 min). The design of the warm water experiment allowed us to follow the expression of every gene under diverse conditions: not exposed (control group), exposed for 2 min (T2) and exposed to 2 min and left afterwards at normal water temperature for 24h (T24), helping in the identification of the best candidates for developing resistance molecular markers. For example, EMLSAT00000004531 gene was up-regulated in resistant T2 lice compared to T2-S (Table 16 and Fig. 8). However, most lice from the sensitive T24 group had that gene also up-regulated, whereas the T24 resistant lice had an expression similar to the control group. EMLSAT00000010792 gene was up-regulated only in the T2 resistant group, which may make this gene a good candidate as marker. EMLSAT00000008147 gene was down-regulated in the T2 resistant group compared to the T2 sensitive lice, however, when plotted all groups together, the expression pattern revealed that the T2-S was up-regulated compared to all the other groups, that did not change the expression compared to the control group. Although some interindividual variation can be seen, this gene might be used as a molecular marker.

Table 16. Gene expression data of the eight genes differentially regulated between the louse groups T2-S and T2-R in the warm water RNAseq study. Arithmetic mean of the normalized transcript counts in the S (sensitive) and R (resistant) groups. Log2FC: log2 fold change; up-regulation is indicated as positive values; down-regulation as negatives. p(adj): p-value for normalized counts (α = 0.05). ENSEMBL *L. salmonis* transcriptome was used in the analysis.

Gene Id	Arithmetic mean		log2FC	p(adj)	Putative annotation
	S	R			
EMLSAT00000008147	42	4	-3,5	0,0004	Unannotated protein
EMLSAT00000001310	656	209	-1,7	0,0017	matrix metallopeptidase
EMLSAT00000003399	45	12	-1,9	0,0032	Unannotated protein
EMLSAT00000011016	4944	1526	-1,7	0,0106	Serine protease
EMLSAT00000006013	404	128	-1,7	0,0480	Protein containing a chitin binding domain
EMLSAT00000000271	368	484	0,4	0,0431	Transcription factor
EMLSAT00000004531	14015	39406	1,5	0,0431	Metallopeptidase
EMLSAT00000010792	8615	11924	0,5	0,0480	Peroxiredoxin

Fig. 7. Number of genes differentially expressed from the warm water RNAseq study. Lines connecting the lice groups show the different group comparisons. Up-regulation is indicated as upward arrows and down-regulation as downward arrows.

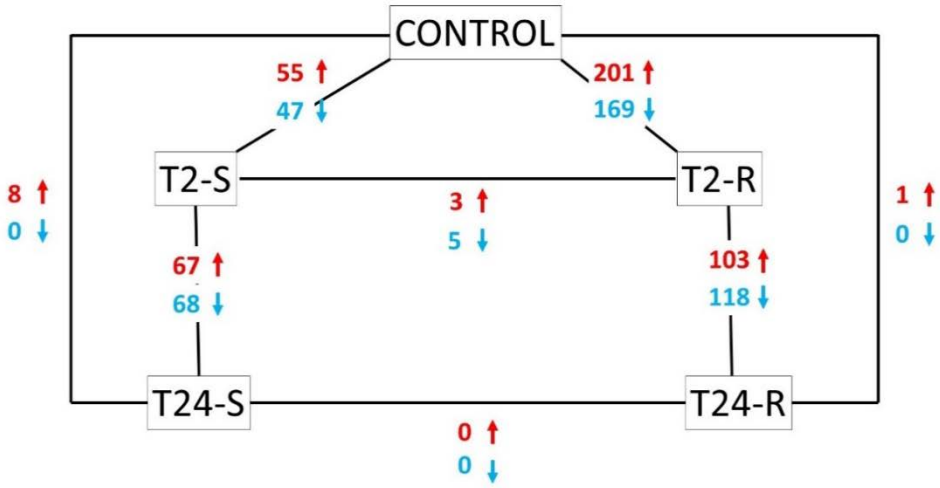
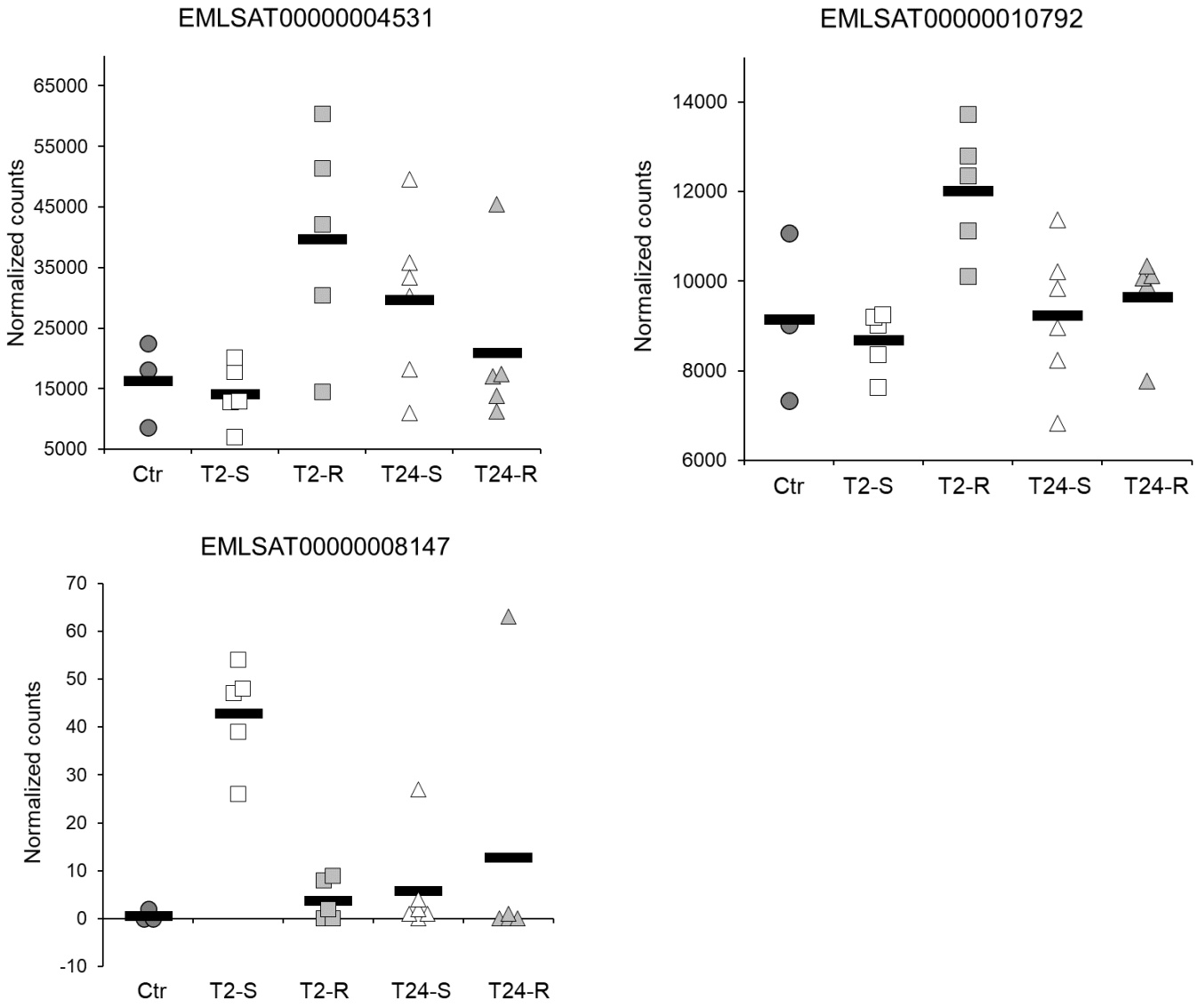


Fig. 8. Gene expression data (normalized counts) of three genes differentially regulated between T2-S and T2-R groups from the warm water RNAseq study. The expression data for the five louse groups included.



RNAseq expression analysis: Freshwater

RNAseq gene expression analysis (DESeq2) for the freshwater experiment showed that there were 44 genes differently regulated between sensitive and resistant lice (Fig. 9 and Table 17): 29 genes up-regulated in resistant lice compared to sensitive lice, and 15 genes down-regulated. The putative annotation of a selected group of genes is shown in Table 17. The DESeq2 analysis cleared separated the two control groups from each other: the bioassay control group (Control-B) and the unexposed lice fixed the same day of collection (Control), with 1.172 genes differently expressed (693 genes down-regulated and 479 genes up-regulated). These differences probably correspond to a louse stress response due to handling, the *in vitro* keeping of the lice (off the fish) for two days, etc. in the control bioassay lice. The Control-B group had 2269 genes differentially regulated compared to the sensitive group, and 577 genes compared to the resistant group. The Control group had 4661 genes differentially regulated compared to the sensitive group, and 2222 genes compared to the resistant group.

The gene expression differences found between sensitive and resistant lice could point to an incipient selection of less sensitive lice to freshwater, and some genes might be candidate genes to develop molecular markers for freshwater resistance. Nevertheless, it is important to stress that those differences were found after the exposure of the lice to freshwater.

As it happened in the warm water experiment, when the number of transcripts of all louse groups were plotted together (both control groups, sensitive and resistant lice), a more precise expression pattern appears (Fig. 10). For example, EMLSAT00000005516 gene was down-regulated in the resistant group compared to the sensitive lice, however, when plotted all groups together, the gene in the sensitive lice was up-regulated compared with all the other groups. EMLSAT00000012109 was an interesting gene as possible candidate for molecular marker since it was downregulated in resistant lice compared to the other three groups. EMLSAT00000005226 gene was up-regulated in the resistant group compared to the sensitive lice, however, the four-groups plot shows that the three bioassay groups were down-regulated compared to lice fixed the same day of collection, probably reflecting a stress-related response. Interestingly, the sensitive lice showed an even lower regulation of the gene than resistant and control-bioassay groups. Although a relatively large interindividual variation, the EMLSAT00000004531 gene was interesting since it was up-regulated only in resistant lice. However, this gene was also differentially regulated in the warm water RNAseq study (see Fig. 8 and Table 16). In both cases, the gene was upregulated in resistant lice when compared to sensitive lice and the control group.

Table 17. Gene expression data of several genes differentially regulated between sensitive (S) and resistant (R) louse groups in the freshwater RNAseq study. Arithmetic mean of the normalized transcript counts. Log2FC: log2 fold change; up-regulation is indicated as positive values; down-regulation as negatives. p(adj): p-value for normalized counts (α = 0.05). ENSEMBL *L. salmonis* transcriptome was used in the analysis.

Gene Id	Arithmetic mean		log2FC	p(adj)	Putative annotation
	S	R			
EMLSAT00000005516	4185	2388	-0,8	< 0.0001	Pyridoxal phosphate-dependent transferase
EMLSAT00000012109	141	66	-1,1	0,00015	Unannotated protein
EMLSAT00000009977	882	578	-0,6	0,049	Histone
EMLSAT00000005226	2808	5996	1,1	< 0.0001	Unannotated protein
EMLSAT00000004531	6277	15814	1,3	0,0007	Metallopeptidase
EMLSAT00000001725	14	51	1,9	0,0018	CHK kinase-like
EMLSAT00000007701	131	330	1,3	0,018	Purine nucleoside phosphorylase
EMLSAT00000007382	489	1054	1,1	0,029	Unannotated protein

Fig. 9. Number of genes differentially expressed from the freshwater RNAseq study. Lines connecting the lice groups show the different group comparisons. Up-regulation is indicated as upward arrows and downregulation as downward arrows. S: sensitive lice. R: resistant lice. Control: Lice fixed the same day of collection from fish. Control-B: Lice used as control bioassay group, kept at 33 ‰ salinity.

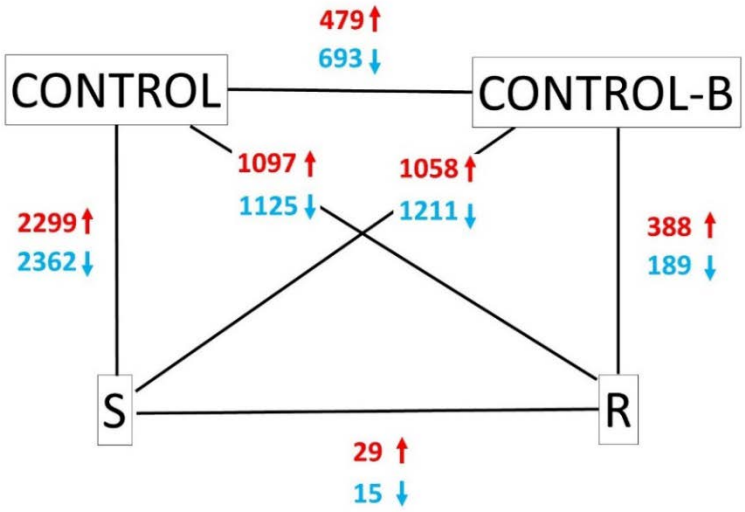
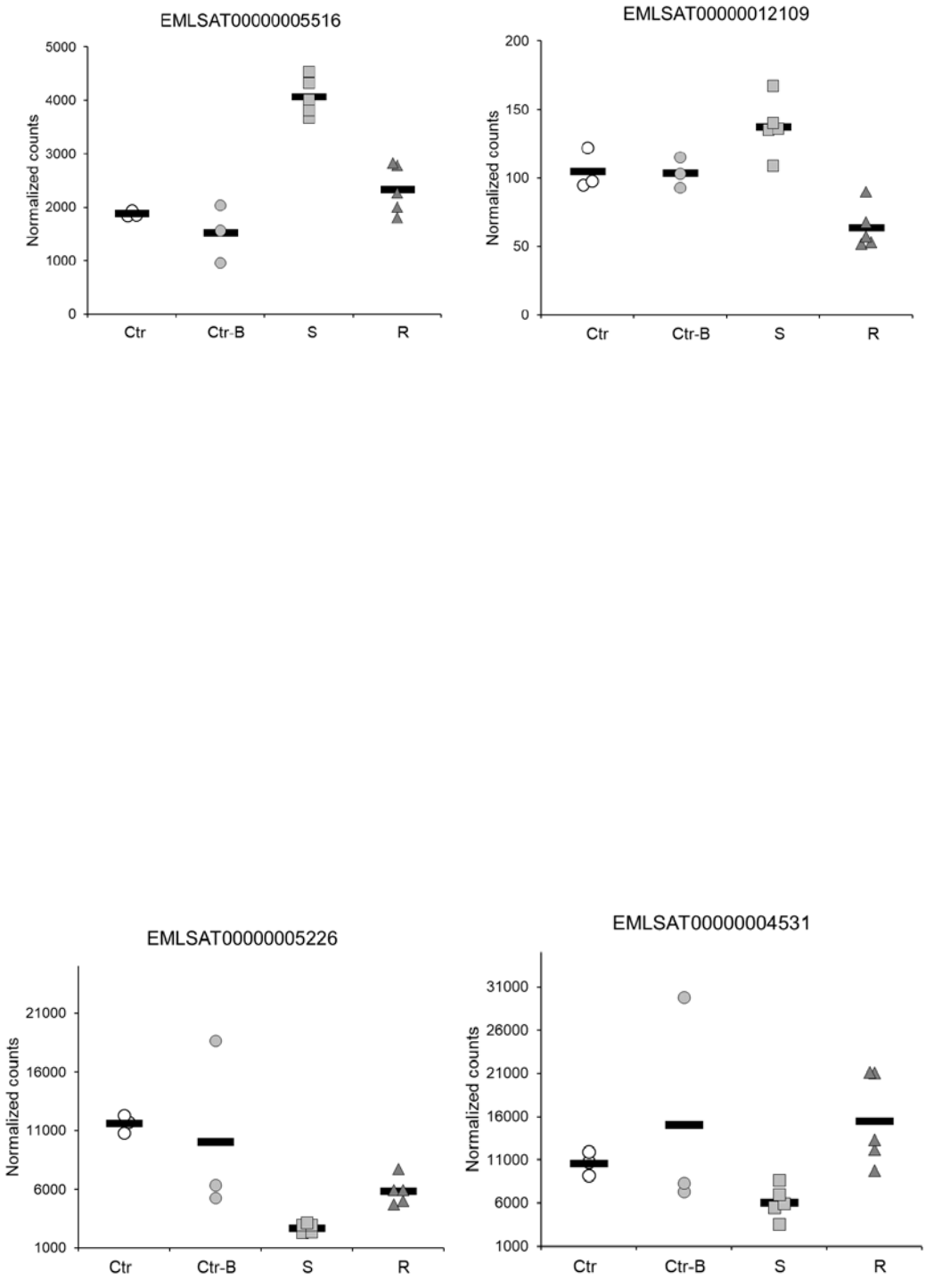


Fig. 10. Gene expression data (normalized counts) of four genes differentially regulated between sensitive and resistant lice from the freshwater RNAseq study. The expression data for the four louse groups included.



Next steps

- To re-analyze the RNAseq data using a *de novo* transcriptome (more complete than the ENSEMBL one). The three control groups will be used for analysing the warm and freshwater data. Warm and freshwater data will be compared to search for shared genes differentially expressed in both treatment types.
- A more thorough search of candidate genes to develop molecular markers for tolerance will be performed using bioinformatics (JExpress).
- A GO enrichment analysis will be performed to find which GO terms (molecular function, biological process, cellular component) are over- or under-represented using annotations for a selected set of genes. This analysis may shed light on the possible tolerance mechanisms.
- Data will be published in a peer-review journal. A patent application will be prepared if interesting candidate genes for tolerance monitoring are found.

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Work package 2:

Goal 1: Freshwater treatment literature review

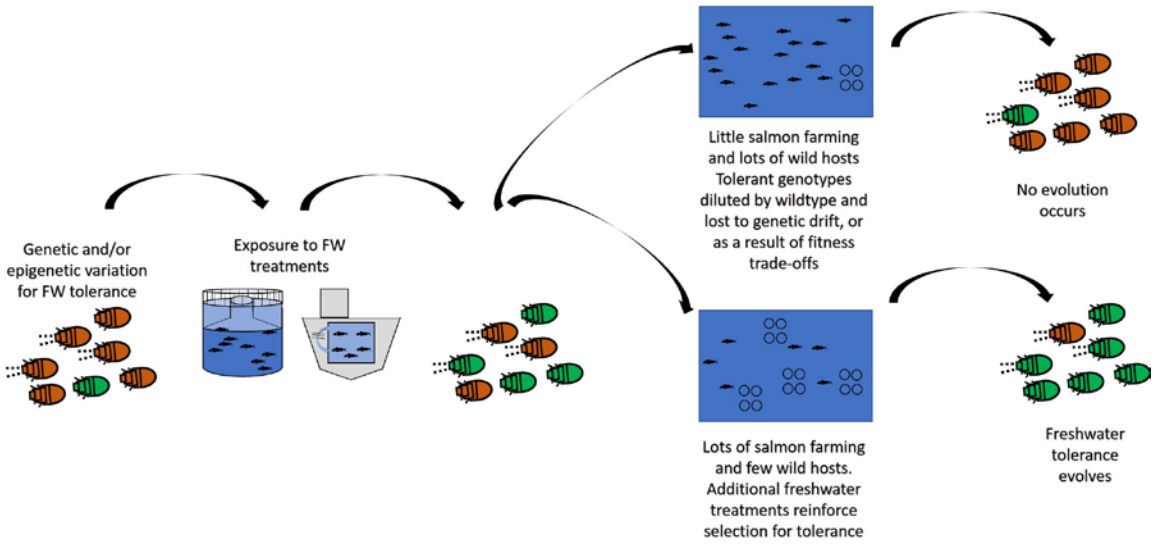
Usage of non-medicinal methods (NMMs) to control salmon louse infestations on salmon farms has raised questions about whether sea lice may be able to evolve tolerance of NMMs. Of concern is the potential for salmon lice to evolve freshwater tolerance as a result of freshwater treatments. Wild trout and some juvenile salmonids swim into freshwater to control infestations and regain ionic balance after disruption by salmon lice; freshwater tolerance would compromise this adaptive behavior.

Here we evaluate the potential for freshwater tolerance to evolve in the salmon louse, *Lepeophtheirus salmonis*. When exposed to low salinity water, parasitic stages of salmon lice can osmoregulate through the host, while larval planktonic stages can not. Transcriptomic work suggests that salmon lice mount a costly polygenic stress response when exposed to brackish water. The population structure of salmon lice is panmictic in both the Pacific and Atlantic Ocean making it conducive to rapid evolutionary responses. It is unknown how much heritable genetic variation these panmictic populations have for freshwater treatments. While usage of freshwater treatments on wellboats is increasing, it is unclear whether the freshwater itself is a strong selective force; in addition to freshwater exposure, wellboats kill lice through physical disruption and filtration of detached lice. Future studies are advised to quantify the heritable variation in freshwater tolerance in salmon louse populations, characterize mechanisms for freshwater tolerance in planktonic and attached salmon lice, and assess the risk of freshwater tolerance evolution under different management strategies. The literature review has been published in Aquaculture Environment Interactions.

Table 1. Mechanisms affecting the evolution of freshwater tolerance and support for ideas

Mechanism	State of knowledge	Relevant literature
(1) Heritable variation in freshwater tolerance	Lab-based evidence for tolerance of brackish water based on 2 populations	Lungfeldt et al. 2017
(2) Population genetic structure	Weak population structure in Atlantic sea lice from wild fish dilute gene pool on farmed fish	Glover et al. 2011, McEwan et al. 2015
(3) Fitness trade-offs associated with freshwater tolerance	Freshwater tolerance slows development and reduces saltwater tolerance in non-parasitic copepod *E. affinis*.	Lee & Petersen 2003, Lee et al. 2007
	Sea louse transcriptome suggests that tolerating less salinated water is energetically demanding.	Sutherland et al. 2012
(4) Selection for increased tolerance of freshwater	Detachment during pumping, filtration and freshwater may all contribute to sea louse death in wellboat treatments	Reynolds 2013, Oppedal et al. 2017

Fig. 2. Flowchart for evolution of freshwater tolerance in salmon lice. In both metapopulation scenarios, a high level of connectivity is assumed to occur.



Goal 2: Freshwater treatment risk assessment

Using the data collected during the literature review, a detailed risk assessment of the appearance of freshwater tolerance in salmon lice was conducted. Consolidating results from a literature search of potentially applicable methods, we proposed a stepwise integrated methods approach that incorporated foundations from an antimicrobial resistance framework, the Office International Epizooties risk model, quantitative microbial risk assessment and infectious disease transmission models. We suggested that an initial ranking profile could be used to prioritize more in-depth qualitative and quantitative risk assessments, when data is available. The risk assessment framework has been published in Aquaculture Environment Interactions.

DELIVERABLES

Work package 1:

Publications

1. Andrews M, Horsberg TE. Sensitivity towards low salinity determined by bioassay in the salmon louse, Lepeophtheirus salmonis (Copepoda: Caligidae). Aquaculture 2020; 514: 734511. https://doi.org/10.1016/j.aquaculture.2019.734511
2. Andrews M, Horsberg TE. In vitro bioassay methods to test the efficacy of thermal treatment on the salmon louse, Lepeophtheirus salmonis. Aquaculture 2020. *In press.*

Work is underway to publish results from the RNAseq section of this project and will deal with both the freshwater and heat treatment sections of this project.

Presentations

The following oral presentations were given at the Sealice 2018 conference that was held in Punta Arenas, Chile in November 2018:

- Andrews, M. & Horsberg, T.E. Freshwater and heat treatment: Developing bioassays to determine treatment efficacy. Sealice 2018. Punta Arenas, Chile, November.
- Andrews, M. & Horsberg, T.E. Developing in vitro bioassay methods to determine the efficacy of freshwater and heat treatment on the salmon louse, Lepeophtheirus salmonis. Sealice 2018. Punta Arenas, Chile, November. (Invited speaker)

The following oral and poster presentations were given at the the European Association of Fish Pathologists conference that was held in Porto, Portugal in September 2019.

- Andrews, Melanie; Horsberg, Tor Einar. Developing bioassays and determining baseline salinity tolerance of salmon lice, *Lepeophtheirus salmonis*, a problem parasite of farmed Atlantic salmon.
- Andrews, Melanie; Horsberg, Tor Einar. Developing bioassays to determine effect of warm water bathing on salmon lice, *Lepeophtheirus salmonis*, a parasite of farmed Atlantic salmon.

The following oral presentation was given at the FHF conference that was held in Trondheim, Norway in January 2020:

- Andrews, M. (2020) Tilpasser lusa seg ferskvann og temperatur? Fiskeri- og Havsbruksnæringens Forskningsfond: Lusekonferansen 2020. Trondheim, Norway, January.

Work package 2:

Publications

3. Groner ML, Laurin E, Stormoen M, Sanchez J, Fast MD, Revie CW. Evaluating the potential for sea lice to evolve freshwater tolerance as a consequence of freshwater treatments in salmon aquaculture. Aquacult Environ Interact 2019; 11: 507-519. https://doi.org/10.3354/aei00324
4. Laurin E, Stormoen M, Revie C, Sanchez J. A stepwise integrated framework for assessing risk in aquaculture based on available information: the case of sea lice tolerance to freshwater treatments on salmon farms. Aquacult Environ Interact 2020. *In press*. https://doi.org/10.3354/aei00373

COMMENTS

In this work package 1 we aimed to increase our understanding of the effects that two commonly used anti-louse treatments, freshwater bathing and warm water bathing, have on the salmon lice. In addition, we aimed to provide protocols allowing researchers and fish health workers to test the local lice populations sensitivity to the planned treatment on site. And finally, RNAseq analyses are currently underway to determine the effect that both freshwater and warm water exposure has on the salmon lice at the molecular level, and to search for potential candidate genes for molecular markers development.

The bioassay protocols designed during this project proved to be highly replicable, in addition they require relatively basic equipment ensuring that they can be conducted in the field. We were able to conduct the same bioassays for all populations included in this project, increasing our knowledge of the baseline tolerance to freshwater and warm water treatments. We found that the copepodid stage tolerate relatively low salinity levels for long periods of time, possibly allowing them to easier spread between regions. And that both the copepodid and pre-adult II stages were initially affected by warm water exposure, yet the majority were able to recover within a few minutes thus leading us to conclude that they were relatively tolerant to short-term warm water exposure. This trend indicates that it is imperative that filtration of the treatment water removes 100% of the detached salmon lice, as we have shown that the majority recover and are able to swim and attach within minutes following short-term exposure. Due to time constraints we were only able to include 7 populations in this project, we suggest that the protocols developed during this project should be used to conduct a more thorough survey from the primary fish farming zones along the Norwegian coastline. This would provide a better overview of the regional treatment sensitivity status and allow for more efficient use of treatments in the future.

Work package 2 dealt freshwater bathing as it is used more frequently in all Atlantic salmon farming regions. A literature review was conducted to determine what is known about the freshwater bathing method as well as what we know about the processes necessary for freshwater tolerance to evolve in salmon lice. Using this acquired knowledge, a risk assessment was conducted dealing with assessing the likelihood of such resistance developing. This work package has succeeded in collecting information regarding freshwater bathing that is often difficult to find and provided very useful information needed in order to better understand how freshwater bathing works and in turn be able to assess whether to use freshwater bathing or not.

This project has provided the groundwork for improving our understanding of the interaction between the salmon lice and two commonly used control methods. The publications resulting from this project that will become available to other researchers and the industry, should assist in improving the knowledge of how these control methods work and to ensure that they maintain their efficacy over time.