SLUTTRAPPORT

OSMO_LUS: Lus i ferskvann

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1. Sammendrag

To gain more knowledge about fresh water tolerance in salmon louse two distinct approaches were undertaken: evaluation of additive effects of a fresh water/approved medicine and identification of the main players (ion pump genes) in osmoregulation in salmon louse.

The evaluation of the additive effects of a combination of approved anti-lice medicines and fresh water was conduced according to the plan. Multi-resistant *L.salmonis* LsH2O2 strain was used in order to evaluate if the osmotic stress caused by fresh water pre-treatment can sufficiently sensitise lice to the tested medicines. Combination treatment did not show any significant increase in lice loss for any of the tested drugs in comparison with fresh water or seawater/drug controls. However, in case of azamethiphos, cypermethrin and H2O2 lice lost from the fish were morbid or dead while fresh water and drug control animals were viable.

A number of ion pump genes involved in osmoregulation was identified in salmon louse and tested if their expression is modulated by fresh water treatment (decreased osmolality). The affected genes were selected for further analysis by RNA interference on larval and adult stages. Three genes were identified having significant role in animal physiology and development. Careful investigation of obtained phenotypes indicates that knock-down of these genes influence animal infectivity, reproduction and survival, making them interesting candidates for vaccines development and drug targets.

These results indicate that influencing the lice's ability to osmoregulate could be an important tool in salmon louse control.

For å få bedre kunnskap om ferskvannstoleranse i lakselus ble det utført to forskjellige tilnærminger: evaluering av additive effekter av ferskvann/godkjente legemiddel og identifisering av hovedspillerne (ionepumpegener) i osmoregulering i lakselus.

Evalueringen av additivvirkningen av en kombinasjon av godkjente antiluselegemiddel og ferskvann ble gjennomført i henhold til planen. Den multiresistente *L. salmonis* LsH2O2-stammen ble brukt for å vurdere om det osmotiske stresset, som følge av forbehandling med ferskvann, kunne tilstrekkelig sensitivere lus mot de testede legemidlene. Kombinasjonsbehandling viste ingen signifikant økning i lusetap for noen av de testede legemidlene sammenlignet med ferskvann eller sjøvann/legemiddelkontroller. Imidlertid, i tilfelle der azametifos, cypermetrin og H2O2 ble brukt, var lus som falt av fisken sykelige eller døde, mens ferskvanns- og legemiddelkontrolldyr var levedyktige.

En rekke ionepumpegener involvert i osmoregulering ble identifisert i lakselus og testet dersom uttrykkingen deres ble endret ved ferskvannsbehandling (nedsatt osmolalitet). De berørte genene ble valgt for videre analyse ved RNA-interferens på larver og voksne stadier. Tre gener som har en betydelig rolle i lusens fysiologi og utvikling ble identifisert. Nøye utredning av oppnådde fenotyper indikerer at å slå ned disse genene påvirker lusenes evne til infeksjon, reproduksjon og overlevelse, noe som gjør dem til interessante kandidater for utvikling av vaksiner og legemiddel mot lakselus. Disse resultatene indikerer at påvirkning av lusens evne til å osmoregulere kan være et viktig verktøy for lakseluskontroll.

2. Innledning

Marine organisms had to develop efficient mechanisms to maintain osmotic balance between internal body fluids and their external environment. Failing to properly regulate the internal osmotic pressure can lead to the serious physiological consequences for the animal. One of the mechanisms to ensure the right osmotic balance is to become isoosmotic with the preferable environment. In the stable conditions, this reduces the energy consumption required for maintaining suitable ion balance. Salmon louse *Lepeophtheirus salmonis* as an example of marine species is isosmotic with its environment. Lice's internal osmolality is around 1000 mOsm/l what is consistent with osmolality of normal seawater (35 ‰). However, lice can be expose to different salinity regimes, when settled on its host and together with it migrate to various environments. During these changes in the salinity, salmon louse is faced with a significant challenge of maintaining the ionic concentrations of the body fluids within appropriate physiological limits. Particularly demanding for this crustacean is osmoregulation when it is located in freshwater environments. Salmon louse has the capacity to maintain a narrow range of internal osmolality in response to a wide range of environmental salinities, but only when it is located on its host. In such situation salt losses are counterbalanced by the active capture of Na^+ and Cl^- and other osmolytes from its host [1]. However, this adaptation is temporary, and after longer exposure to lower salinity lice osmoregulation become less efficient and can eventually lead to death.

Adaptation to the new salinity requires metabolic changes, causes osmotic stress and affects animal's ability to survive. Studies on different crustacean species have identified a variety of genes related to osmotic stress, including ion transport enzymes (ion pumps) and amino acid transport proteins. Number

of ion pumps has been identified and their function in pH homeostasis, cell volume regulation and regulation of ion balance have been indicated [2] [3] [4] [5] [6] [7] [8] [9]. As main players in the ion exchange in crustaceans vacuolar H^+ ATPAse (VHA), Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ symporter or a Na^+/H^+ exchanger (NHE) [10] have been suggested. A number of studies approaches the osmoregulation problem in free-living crustacean, however not much is known about osmoregulation of ectoparasites like salmon louse. Detailed knowledge about osmoregulation in salmon louse and identification of the main ion pumps involved in the process can be an important tool in the fight against lice. Because of a high variability in ion pumps between aquatic species, a number of these proteins could constitute good targets for drug development or vaccine targets against salmon louse.

Currently, the negative influence of fresh water on salmon louse is used in the fish farming industry as an important factor in the control of salmon louse, especially in case of multi-resistant lice. Fresh water itself has relatively low effect and requires intensive handling of the fish in order to achieve a satisfactory lice loss. A pilor study conducted by SLRC in 2015 showed that already 4h fresh water treatment is sufficient to induce a low level of lice loss. This suggests that the osmotic stress is already induced in lice and is likely to increase the susceptibility of lice to other treatment agents, for example chemical treatment. Thorough investigation of effects obtained by the fresh water pre-treatment followed by an exposure to the commercially available drugs can provide valid information and new strategy for lice control, especially in the light of spreading resistance and lack of new sustainable treatment tools.

Project organization:

The project was carried out as a project related to Sea Lice Research Centre of the University of Bergen as contracting party. The project was implemented in collaboration with Lerøy Seafood Group ASA and Marine Harvest ASA. SLRC had a project management responsibility for the project. project was prepared by Professor Frank Nilen/director of SLRC and Dr. Anna Z Komisarczuk. Anna Z Komisarczuk was the project leader, responsible for executing all experiments and reporting.

The steering group for the project was:

Gordon Richie (Marine Harvest)

Bjarne Reinert (Lerøy Seafood Group ASA)

Frank Nilsen (SLRC / UIB)

These parties have been in close contact during the whole project.

Scope:

The project has started on 01.02.2016. Experiments were finished in February 2017 and the period after termination until submitting this report was used for analyse of the obtained results.

3. Problemstilling og formål

The goal of this project was to gain more knowledge about fresh water tolerance in salmon louse. The project was divided into two parts:

1. Effects of a combination of fresh water and approved medicines. The aim of the experiments conduced in this part was to determine if lice pre-treated with fresh water are more sensitive to medicinal treatment and if the combination of fresh water and medicinal treatment can be considered in a industrial delousing.

2. Identification of ion pumps involved in osmoregulation in salmon louse. The aim of this part was to identify ion pump genes, which are crucial for an animal to osmoregulation and survive negative effects of lower osmolality (brackish water, salmon blood). Experiments were conducted at adult sea lice set on fish and on *L.salmonis* larval stages. Identified genes were analysed further as a potential candidate for anti-lice treatment.

4. Prosjektgjennomføring

Part 1.

Fresh water and drug treatment

Multi-resistant strain of Salmon lice *Lepeophtheirus salmonis Ls*H2O2 was used in this study. Prior to the experiment, 8 females and 8 males were placed on the fish (Atlantic salmon, *Salmo salar*) and allowed three days to acclimatise to the new conditions (Figure 1). Unspecific lice loss was monitored every day. Both lice and fish were kept in seawater with a salinity of 34.5 ‰ and a temperature of approximately 12 °C.

For each drug/fresh water test four groups of fish (three fish per group) were used to control influence of each agent. Two groups were treated with fresh water (FW), and one of them was additionally exposed to the drug (FW+D). Two other groups were treated only with seawater (SW) and one of them additionally was exposed to drug (SW+D). Experimental groups used in each experiment are shown on Figure 1A.

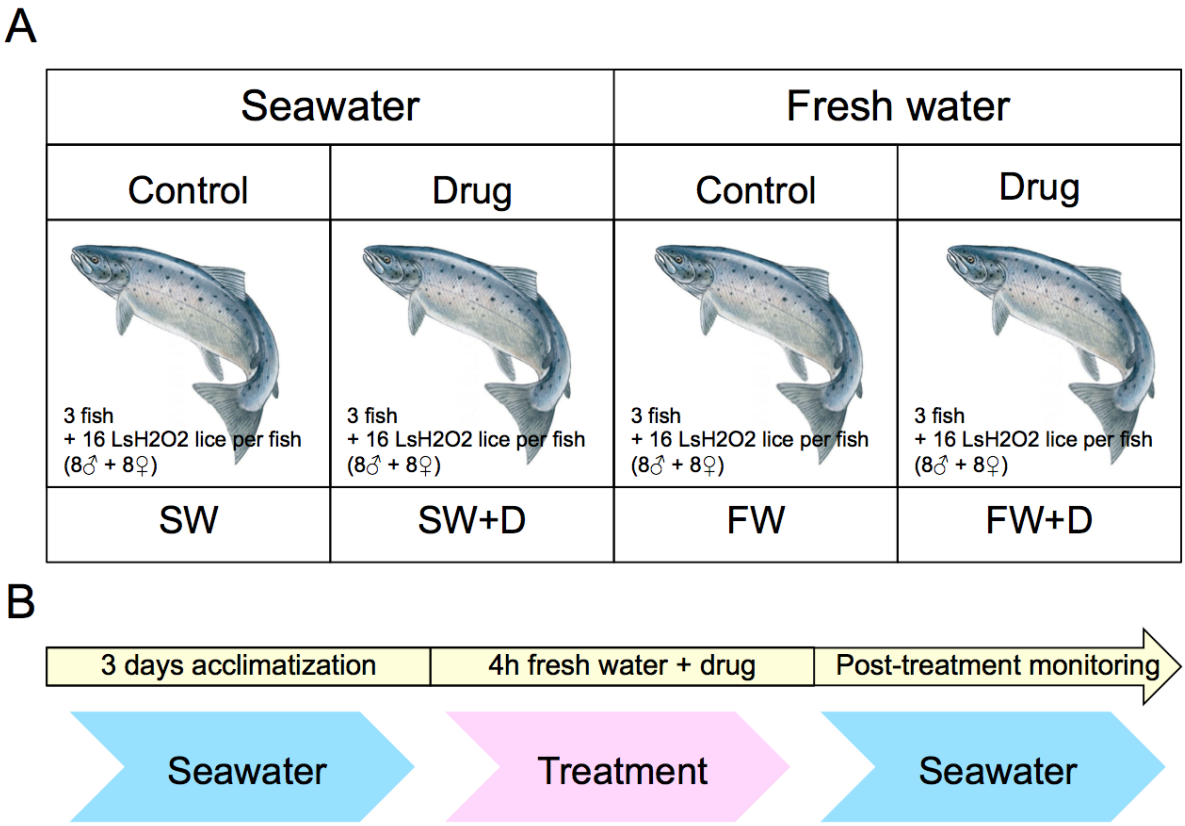

Figure 1. Experimental groups used during test of each fresh water/drug combination.
A. Experimental groups used in each experiment. Using all 4 combinations of tested agents (fresh water/seawater, drug/no drug) allows controlling effects of each agent used. B. Experiment timeline. Lice were given three days for acclimatization. Lice loss effects were observed for three days post treatment. SW – seawater only, SW+D – seawater and drug, FW – fresh water only, FW+D – fresh water and drug

The experiments began with fresh water pre-treatment. By measuring the salinity of water that was leaving tanks, the salinity changes were closely monitored. First, fish/lice were treated with fresh water (3h – 3h 40 min depending on the tested medicine), and at the end of this period water flow was stopped, water level in each tank was adjusted to 50 litres and air was provided to each tank by air stones. Proper dose of the medicine previously diluted in fresh water was added to each tank, above the air stone, in order to achieve fast and ubiquitous distribution in the tank. Fish/lice were treated with fresh water/drugs for 20 to 60 minutes. Dose of medicine and treatment time are according to veterinary recommendations and summarised in Table 1.

Table 1. Approved medicine used in the course of experiments. All medicines were used as a batch treatment, except emamectine benzoate, which was given as an injection. Table contains information about final concentration of the active compound during treatment.

Medicine name	Active compound	Final concentration used	Bath time
Betamax	Cypermethrin	0,015 mg/l	30 min
Alpha max	Deltamethrin	0,002 mg/l	30 min
Salmosan	Azamethiphos	0,1 mg/l	60 min
Paramove	H2O2	1500 mg/l	20 min
Paramove	H2O2	1000 mg/l	20 min
Aqui-S	Eugenol	5 mg/l	60 min
	Emamectin benzoate	100 µg/1kg – injection	7 days before lice exposure

Lice loss was monitored closely during the whole experiment. Lost lice were collected from the nets fixed under the water outlets every 30 minutes during experiment and once a day for three following days. On day 3 post-treatment, remaining lice were removed with forceps and inspected under a binocular microscope (Olympus SZX12, 0.5× Olympus objective) for abnormalities.

Part 2.

Fresh water treatment of adult lice – time series

Experiment was conduced in similar manner as in the fresh water/drug treatment described in Part 1, but with some modifications. Briefly, only two experimental groups were used, each containing 6 fish: treatment group (FW) was exposed to fresh water for 6 hours, while a control group (SW) was exposed only to seawater.

Multi-resistant strain of Salmon lice *Lepeophtheirus salmonis Ls*H2O2 was used in this study. Prior to the experiment, 8 females and 8 males were placed on the fish (Atlantic salmon, *Salmo salar*) and allowed three days to acclimatise to the new conditions. Unspecific lice loss was monitored every day. Both lice and fish were kept in seawater with a salinity of 34.5 ‰ and a temperature of approximately 12 °C.

Experiment started with changing seawater to fresh water in FW group tanks. By measuring the salinity of water that was leaving tanks, the salinity changes were closely monitored. Water salinity of 0 ‰ was consider as a start of the experiment. Lice were sampled every hour from the anaesthetised fish and placed to RNAlater (Ambion) for further analysis. Lice loss was monitored closely during the whole experiment. Lost lice were collected from the nets fixed under the water outlets every 10 minutes during the whole experiment and preserved in RNAlater (Ambion).

Incubation of copepodids in various water salinities

Free-living copepodids (7 days post hatching) were divided into 3 groups, each incubated in different water salinity: 34,5 ‰, 27,6 ‰, 20,7%, 13,8% and 6,9 (100%, 80%, 60%, 40% and 20% sea water respectively). Water was prepared by mixing seawater with fresh water in a required proportion and the

final salinity was evaluated with a salinity sensor. Water temperature was maintained at 10°C during the course of the experiment. Copepodids (around 200 per group) were placed in 50 ml Falcon tubes and water of a specified salinity was added. Animals were kept without water flow; therefore water was changed every 4 hours in order to maintain a sufficient oxygen level. Animals were collected every 4 hour during the first day and once a day for next 2 days and stored in RNAlater for subsequent total RNA extraction. Three parallels were prepared for each time point and salinity.

RNA interference

To test physiological function of ion pumps we applied an RNA interference approach. dsRNA for each tested gene was produced from PCR products from MEGAscript® RNAiKit (Ambion Inc.) according to manufacturer instructions. Final concentration of dsRNA was measured with spectrometry (NanoDrop Technologies Inc.) and adjusted to 1.5 µg/µl. RNAi was performed as earlier described (Eichner et al., 2014). The existence of any unspecific effects of dsRNA on gene expression was assessed by treating animals with control dsRNA, containing a fragment with no significant sequence similarity to salmon louse (a cod trypsin gene, *CPY185*) (Dalvin et al., 2009).

RNAi on nauplii I: In brief the experiment was performed using experimental group consisting of approximately 600 nauplius I larvae. For soaking, these were divided in subsamples of 100 animals, each incubated in 200 µl of seawater in the lid of a 1,5ml Eppendorf tube (Eppendorf) where 2 µg dsRNA was added – final concentration of dsRNA 20ng. Animals were incubated overnight for around 20h. Thereafter, all animals within a treatment group were pooled and transferred to a flow through well, where they were kept until sampling in RNAlater (Qiagen). Lice were inspected under a binocular microscope (Olympus SZX12, 0.5× Olympus objective). Photographs were taken of copepodids using a Canon EOS 600D camera mounted with an adaptor (LMscope) to an Olympus SZX9 dissecting microscope.

RNAi on preadult II females: dsRNA was diluted to a concentration of 600 ng/µl and bromophenol blue was added to visualize successful injections. On the day of injection, preadult II female lice were removed from the fish, injected with dsRNA solution, incubated in seawater for 4 h and then placed back on fish together with an equal number of adult males. For each dsRNA fragment, three fish – each with 10 dsRNA-injected females and 10 non-injected males – were used. The experiments were terminated after 30 to 32 days, when all females in the control group had developed into fully mature adults and extruded at least their second pair of egg strings.

RNA extraction and cDNA synthesis

Briefly, animals were transferred to 1 ml of Tri Reagent (Sigma Aldrich) and homogenized in TissueLyser LT (Qiagen). 1.4 mm zirconium oxide beads were used for homogenization of copepodid, and 3 mm stainless steel beads for adult of both sexes. The following steps were according to Tri Reagent manufacturer instructions. Total RNA was diluted in DEPC-treated water (Invitrogen) and the final concentration was evaluated with spectrometry (NanoDrop Technologies Inc.). DNase treatment (TURBO, Ambion) and cDNA synthesis (AffinityScript qPCR cDNA Synthesis Kit, Stratagen) were

performed directly. For DNase treatment each sample contained 2 µg of total RNA, according to NanoDrop readings. cDNA was diluted four times before storage at -20^{o}C.

Histology

Any phenotypic changes caused by RNAi were assessed by histological analysis. Plastic sections: Copepodids and adults of both sexes were fixed in Karnovsky's fixative, and embedded in Technivit7100 plastic according to the manufacturer instructions. 2 µm thick sections were produced using a microtome (Leica RM 2165), stained with toluidine blue and mounted using Mountex (Histolab Products).

In situ hybridization

Expression of analysed genes was evaluated by in situ hybridization on paraffin sections. Salmon lice copepodids and adults were fixed in paraformaldehyde before paraffin embedding. Digoxigenin (DIG) labelled antisense and sense RNA probes of each gene were prepared by in vitro transcription using the DIG RNA Labelling Kit (Roche), with purified PCR products including T7 promoters as templates. In situ hybridisation was carried out as previously described by [11], with the following modifications: Xylene was replaced by Histo-Clear (National Diagnostics) in the dewaxing step. The probe concentration in the hybridisation mix was 20 ng/µl and approximately 100µl hybridization mix was used per section. Chromogenesis was carried out using nitroblue tetrazolium (NBT, Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche). Hybridisations with sense probe were carried out as negative control.

5. Oppnådde resultater, diskusjon og konklusjon

Part 1 – Effects of a combination of fresh water and approved medicines

Azamethiphos (Salmosan)

All female lice were lost from the fish treated with fresh water and drug (FW+D) within 24h after treatment. Animals were morbid or dead. Female lice treated with combination of seawater and drug (SW+D) were lost from the fish within 2 days post treatment, and all animals were viable (Figure 2).

Loss of the males from the FW+D group was similar to this observed in FW control. However all FW+D males were lost within 24h post treatment, while few males from FW control group were still on the fish at the end of experiment. Males from SW+D group were lost from the fish within 2 days post treatment (Figure 2). Males from FW+D group were morbid or dead, while males from SW+D group were alive.

Drug exposure (D, SW+D, FW+D) did not have any visible effect on egg string development and hatching.

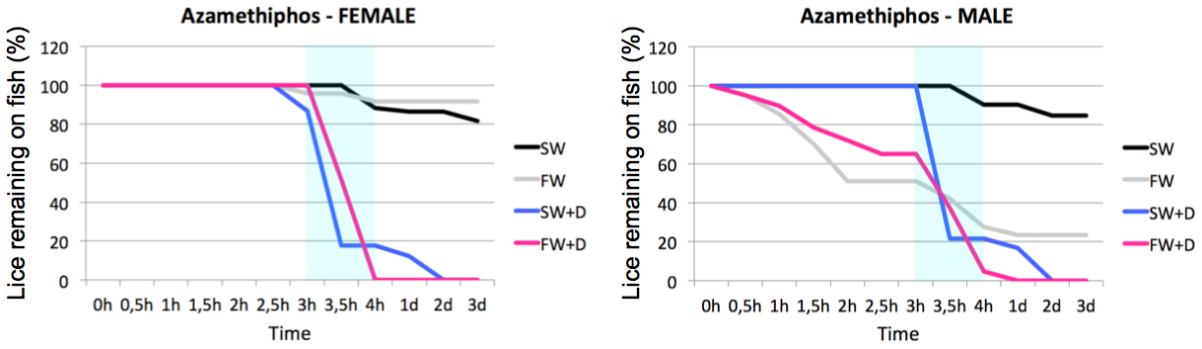

Figure 2. Fresh water and Azamethiphos (Salmosan) – 4h.

Delousing effect cause by Azamethiphos was promising; therefore treatment was repeated with shorter fresh water pre-treatment time. Effects of standard treatment of fresh water (total 4h) and shorter time (total 2h) were compared.

Delousing effects are similar for both cases – 2h and 4h fresh water treatment (Figure 3). Lice loss (both females and males) is intensified by addition of the drug only in 4h fresh water treatment, however similar effect is observed in the group SW+D. Lice lost after combined treatment of FW+D for 4h are morbid and/or dead, while lice treated for 2h do not vary from control animals (both FW and SW).

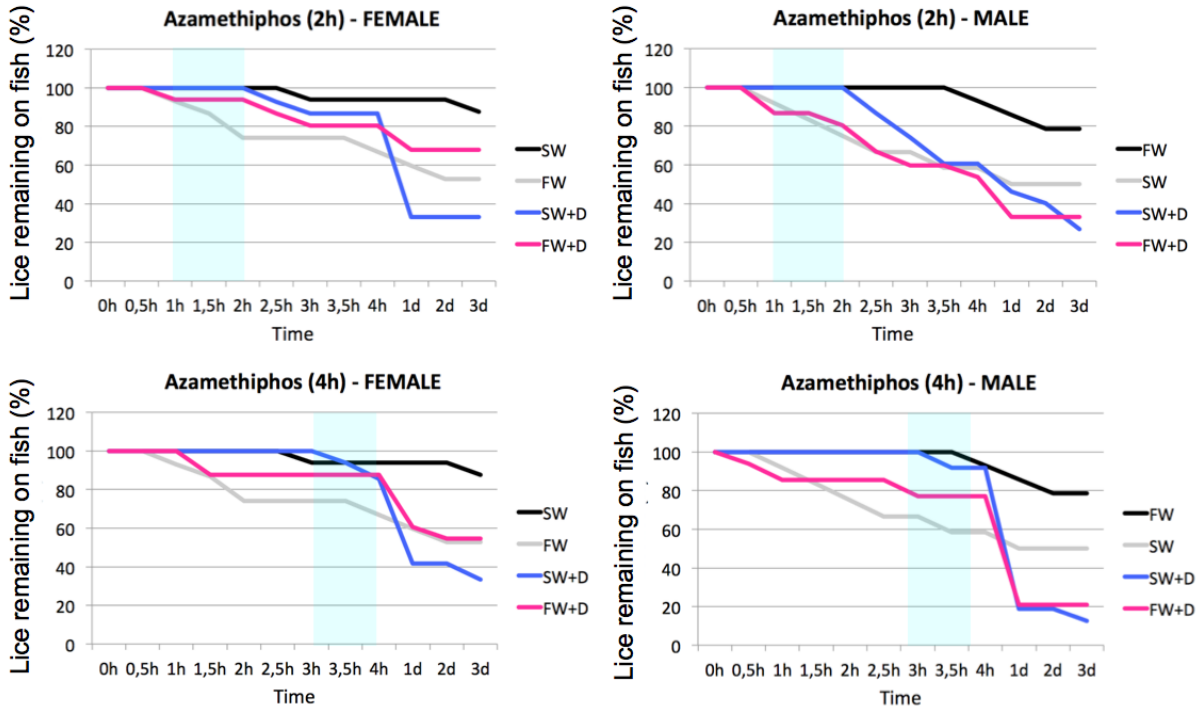

Figure 3. Comparison of the Azamethiphos (Salmosan) treatment combined with various fresh water treatment – total fresh water time 2h and 4h.

Results indicate that *Ls*H2O2 lice are sensitive to the Azamethiphos (Salmosan). Addition of the drug has similar delousing effect on the lice treated with FW and SW, however animals treated with FW+D, in 4h experiment, are morbid or dead, while animals from SW+D group were still alive. This decrease in viability is not observed in case of shorter fresh water pre-treatment. Females and males from both groups FW+D and SW+D reveal very similar loss, which is triggered by addition of the drug. This suggests that the addition of the drug rapidly removes sensitive lice (both in FW and SW), while more resistant are still on the fish on day 3 post-treatment. Given the lice condition after FW+D treatment, longer post-treatment observation could show greater delousing effects.

The best additive effects of fresh water and drug combination were observed for Azamethiphos, therefore this FW+D combination was tested also on resistant lice from the field (Hjartholm, Marin Harvest).

Azamethiphos has a visible effect on both females and males (both SW+D and FW+D). Loss of lice in FW, FW+D and SW+D is similar for both sexes and no enhanced lice loss is observed in combination of fresh water and Azamethiphos (Figure 4). Lice lost after combined treatment of FW+D were morbid or dead, while lice from SW+D group were viable. No significant effect of FW+D or D was observed on egg strings.

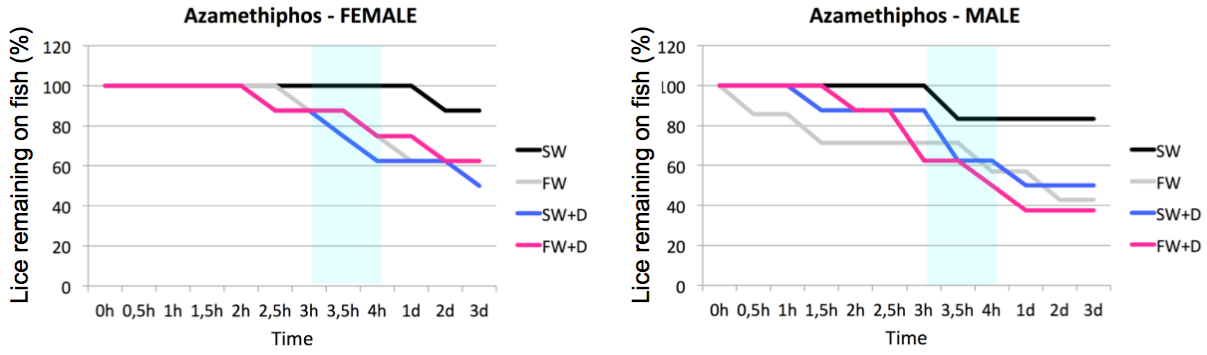

Figure 4. Treatment of lice obtained from Hjartholm (*Ls*Hjartholm). Fresh water and Azamethiphos (Salmosan) – 4h.

Deltamethrin (Alpha max)

Loss of female lice was very similar in all treatment groups. Deltamethrin has no effect on female lice regardless of combination with SW or FW (Figure 4). Drug exposure did not have any visible effect on egg string development and hatching.

Deltamethrin treatment has no effect on male lice. Loss of male lice in these groups is connected with fresh water treatment (Figure 5).

Lice from *Ls*H2O2 strain are resistant to Deltamethrin (Alpha max) and fresh water treatment does not enhance/cause effect of the drug.

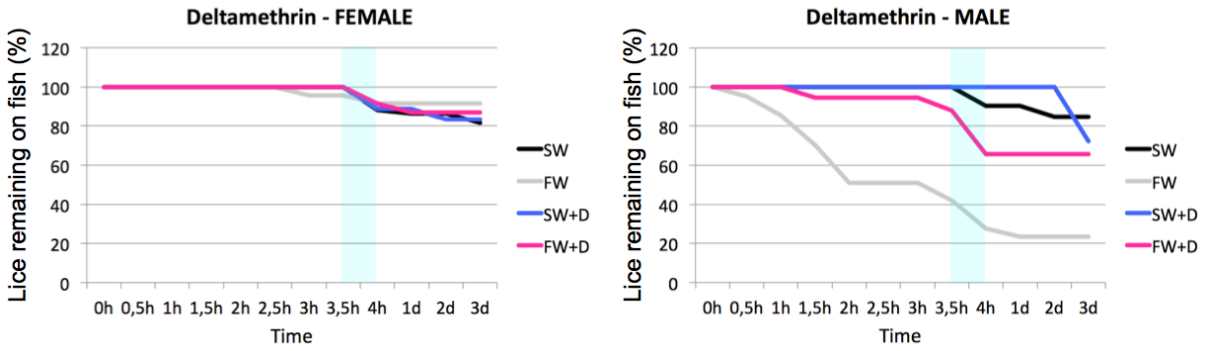

Figure 5. Treatment with combination of fresh water (4h) and Deltamethrin (Alpha max).

Cypermethrin (Betamax)

Loss of female lice is connected with fresh water treatment only, and similar loss was observed in lice treated with FW with and without drug. Cypermethrin has no effect on female lice regardless of combination with SW or FW (Figure 6). However, FW+D females lost from the fish during the experiment were morbid or dead, while female lice from FW and SW+D groups were alive. This suggests that combination of FW+D cause mortality in the lice that fall from the fish (unspecific loss) but does not cause loss of the lice itself. Drug exposure did not have any visible effect on egg string development and hatching.

Loss of the male lice is cause mostly by fresh water treatment (Figure 6). FW+D males lost from the fish during the experiment were morbid or dead. Male lice from FW group were alive.

Lice from *Ls*H2O2 strain are resistant to Cypermethrin (Betamax). However, fresh water treatment before addition of the drug can enhance lethal effect of the drug in lice, and mildly enhance loss of male lice.

Females and males treated with combination of FW+D were morbid or dead, while animals treated with FW or SW+D did not shown any signs of decreased viability. This suggests that combination of FW+D could cause mortality in the lice that fall from the fish (nonspecific loss) but does not cause loss of the lice itself. Longer post-treatment observation time would be recommended to investigate if FW+D combination can have enhanced delousing effect.

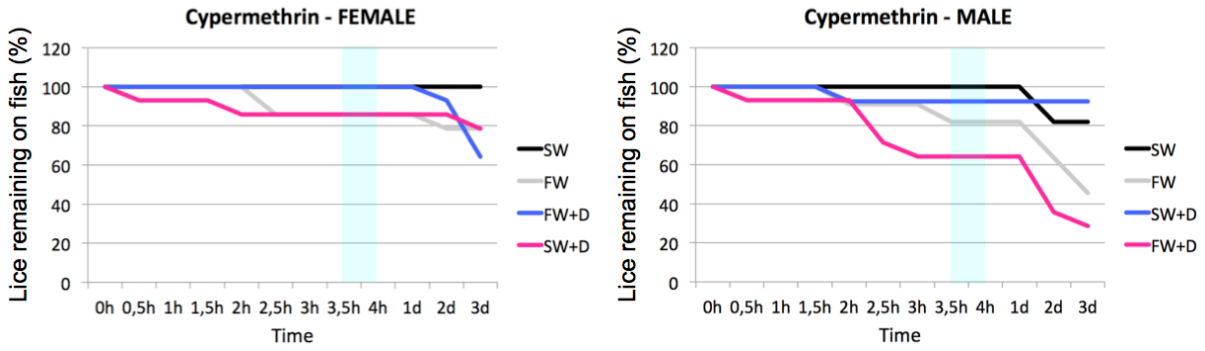

Figure 6. Treatment with combination of fresh water (4h) and Cypermethrin (Betamax).

Eugenol (Aqui-S)

Loss of female lice is connected with fresh water treatment only, and similar loss was observed in lice treated with FW with and without drug. Eugenol has no effect on female lice regardless of combination with SW or FW (Figure 7). Drug exposure did not have any visible effect on egg string development and hatching.

Similar result is observed in male lice. Loss of male lice was connected with fresh water treatment only, and similar loss was observed in lice treated with FW with and without drug. Eugenol has no effect on male lice regardless of combination with SW or FW (Figure 7).

Eugenol (Aqui-S) does not cause loss of *Ls*H2O2 lice. Lice loss in this treatment is connected only with FW.

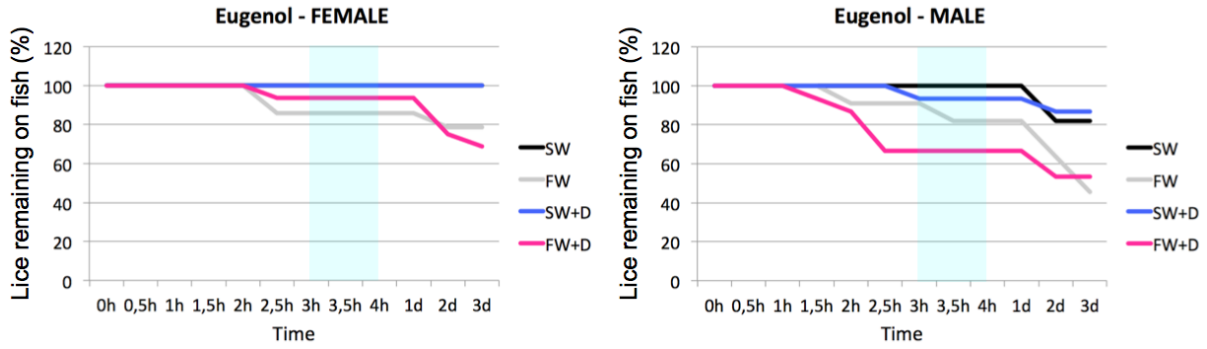

Figure 7. Treatment with combination of fresh water (4h) and Eugenol (Aqui-S).

Emamectin benzoate

Emamectine benzoate was administrated by injection 10 days before start of the experiment. Lice were placed on fish 4 days before treatment. Fresh water treatment started when loss of lice stabilized. No visible effect of drug on females and males was observed – lice loss was connected mainly with fresh water treatment (Figure 8). No additive effects of FW and D were observed. No effects of FW+D treatment on egg strings.

To evaluate if the concentration of ememactine benzoate in fish was sufficient to cause effect on lice, fish tissues were send for analysis.

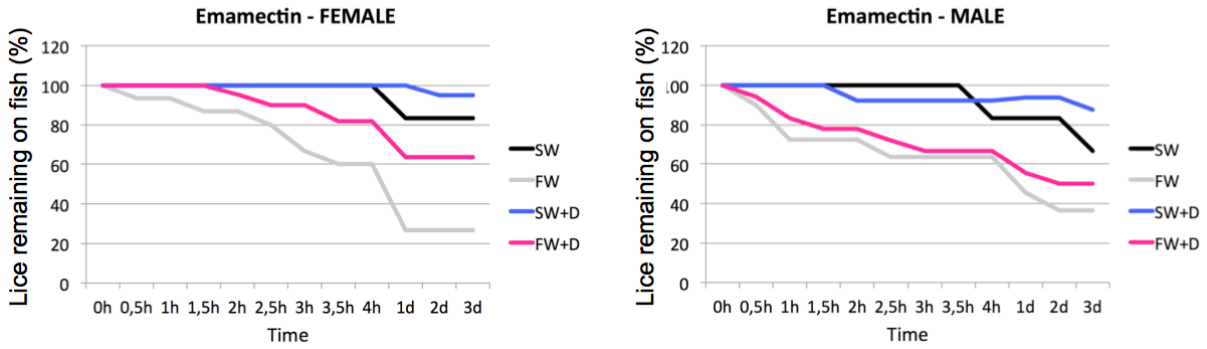

Figure 8. Treatment with combination of fresh water (4h) and Emamectine benzoate (injection).

H2O2

Combination of 1500ppm H2O2 with fresh water causes significant stress to the fish. The experiment had to be terminate 5 minutes after addition of H2O2. However, loss of lice during following days was observed according to the protocol.

All female lice were lost from fish within 24h post treatment. High loss was observed also in the SW+D group, however 20% of female lice were still on the fish after three days. Addition of the drug triggered female loss during experiment (Figure 9). 50 % of females from both drug treated groups (FW+D and SW+D) were morbid or dead and remaining were alive. H2O2 has effect on egg strings. Nearly 100% of egg strings collected during experiment had patches of dead embryos. However, only 20% of egg strings failed to hatch. In the remaining eggs stings hatching success was 15 – 30% decreased (number of nauplii hatched). Nauplii hatched looked and developed normally.

All male lice were lost from fish in the FW+D group and in SW+D group within 24h post treatment. This indicates that animals used in this experiment from *Ls*H2O2 strain are sensitive to H2O2. Fresh water treatment before drug addition caused intensive male loss in FW+D group. Lice loss was triggered by addition of the drug in SW+D group (Figure 9). 70 % of males from both drug treated groups (FW+D and SW+D) were morbid or dead and remaining were alive.

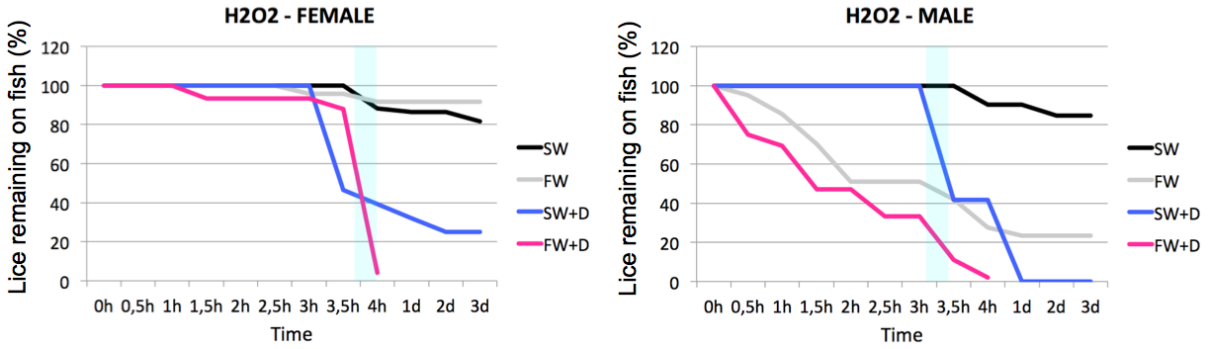

Figure 9. Treatment with combination of fresh water (4h) and H2O2 – 1500ppm (Paramove).

Experiment was repeated with lower H2O2 concentration – 1000ppm. During treatment fish from FW+D did not show any distress sign and experiment proceed according to the plan.

H2O2 itself has a visible effect on both females and males (SW+D). Loss of lice in FW, FW+D and SW+D is similar for both sexes and no enhanced lice loss is observed in combination of fresh water and H2O2 (Figure 10). Females and males lost from all groups were viable. No significant effect of FW+D or D was observed on egg strings.

Batch of LsH2O2 lice used in this treatment seems to be sensitive to H2O2. The standard dose of H2O2 in combination with fresh water is toxic for fish. Lower dose, to avoid negative effects on the fish, has weaker delousing effects. No additive effect was observed of fresh water and H2O2 during 3 post-treatment days.

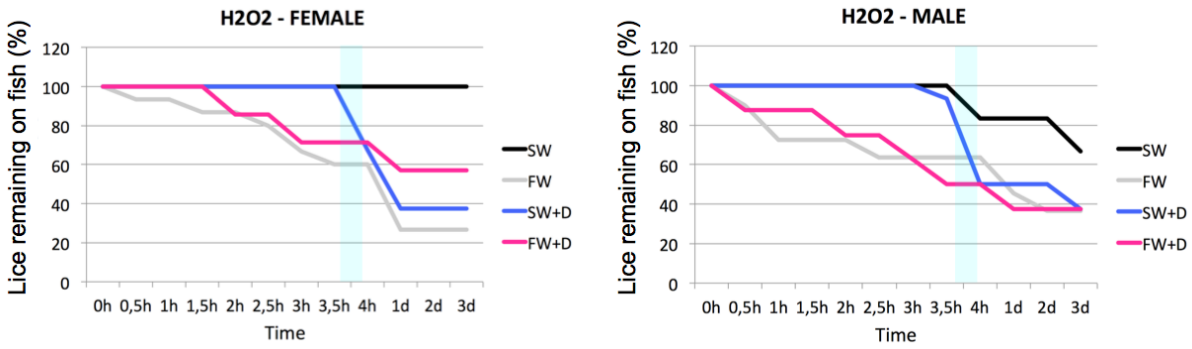

Figure 10. Treatment with combination of fresh water (4h) and H2O2 – 1000ppm (Paramove).

General conclusion Part 1:

The LsH2O2 strain is the laboratory strain, which was tested for resistance, against many drugs used in the industry. It was named LsH2O2 because of the resistance presented against this drug. However, *Ls*H2O2 is not genetically clean strain and variation can be observed also in case of the resistance. All experiment presented here were performed in different time points and different batches of animals from LsH2O2 strain were used. This is likely the reason for observed variation in the results and sensitivity observed against different drugs. In the future, these experiment should be repeated with lice strain which is genetically preselected and tested (genotyped) for resistance against each used drug.

Report – Part 2 – Genes involved in Osmoregulation

Results from this part of the OSMO-LUS project are presented very generally, without any specific information. Data obtained during investigation of ion pumps are now organised into publications. Articles with detailed information about tested genes will be added to this report when published.

Fresh water treatment – adult lice.

Lice samples for evaluation of expression of genes involved in the osmoregulation were collected from fish treated with fresh water for 6h. Control samples were collected from fish treated with seawater. Samples were collected every hour (one fish from each group) and lost lice and egg strings were collected every 10 minutes. The highest loss of lice was observed during first 2 hours of the experiment (Figure 11).

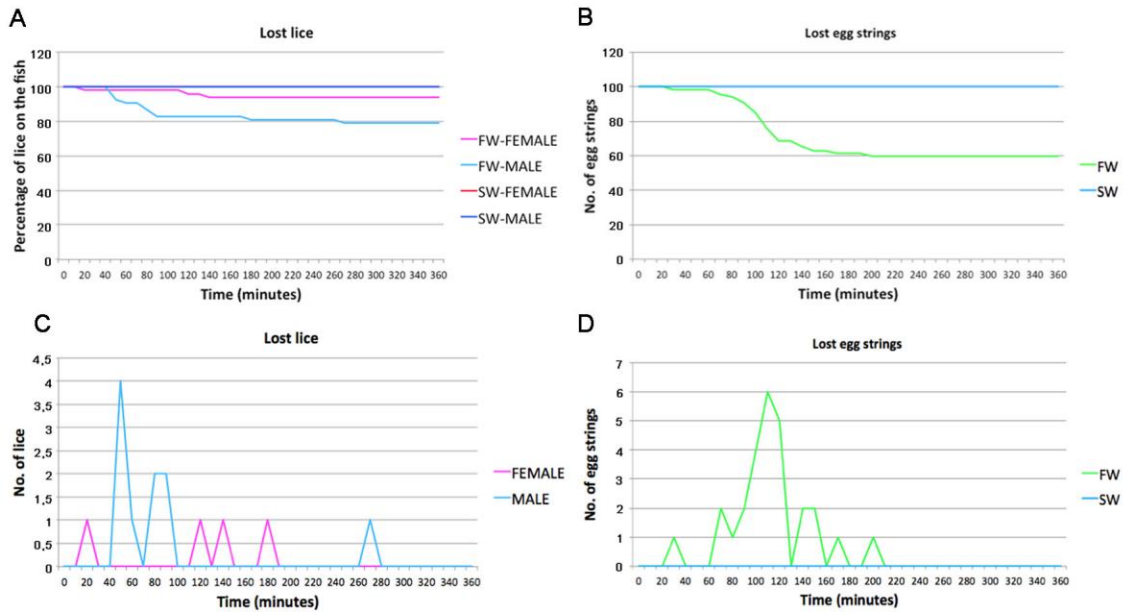

Figure 11. Lice and egg strings loss during 6h water treatment. A and C – adult lice, B and D – egg strings. A and B – the percentage of lice and egg strings present on the fish in each time unit (registered every 10 minutes). C and D – number of lice and egg strings lost in each time unit. No lice and egg strings were lost from control group treated with seawater. A and B – more males were lost from the fish than females. The highest lice and egg strings loss occurred during 3 first hours of the experiment indicating that adaptation mechanisms to lower salinity became activated.

Identification of ion pump genes and osmotic stress-response genes important in salmon louse osmoregulation.

We used ion pump genes and stress response genes of other Arthropods to identify putative genes involved in osmoregulation and osmotic stress in salmon louse. Arthropods genes were selected based on the literature search. Salmon louse ion pump genes were identified by BLAST search, using the salmon louse genome sequence in LiceBase. 21 putative ion pumps genes and 12 osmotic stress response genes were identified in *L. salmonis* and their DNA end protein sequences extracted and analysed.

Expression of ion pump genes and osmotic stress-response genes in adult lice treated with fresh water.

Changes in the expression of ion pumps and stress response genes were evaluated in adult lice (males and females) treated with fresh water for 6h. Expression of many ion pumps changed in the fresh water condition and the highest transcript levels was observed 2h after fresh water treatment started. Lice lost from the fish shown a lower expression of the tested genes compared to lice collected from the fish, indicating that they were not able to adjust to the new conditions (Figure 12). Stress response genes were up regulated in the similar patter as ion pumps but their higher expression lasted longer then ion pumps.

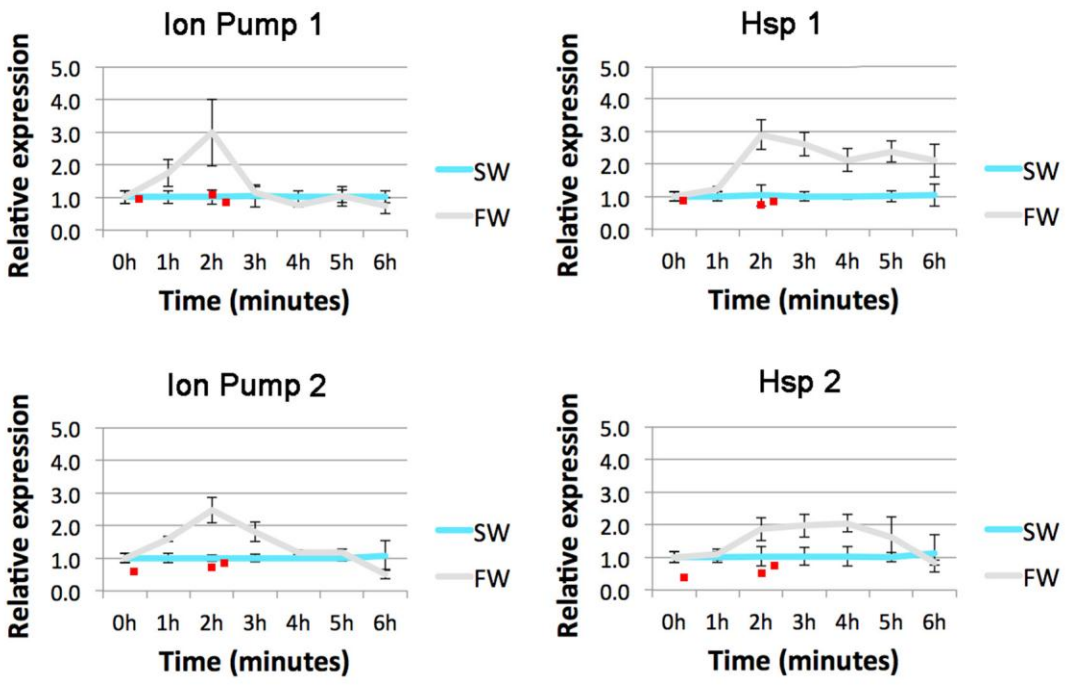

Figure 12. Gene expression changes after fresh water treatment in adult lice collected from fish. Blue – control lice in seawater, grey – animals treated with fresh water, red spots – expression in the animals lost from the fish during experiment – their position indicate the time point when they were lost.

Expression of ion pump genes and stress response genes in copepodids treated with various salinities.

Expression of many of the tested ion pumps and stress response genes increase in reduced salinity. The up-regulation is observed 4h post treatment and increases with time. Transcript level for many genes was still higher than in the controls 72h after treatment (data not shown). This observation is in contrast to changes observed in adult lice where expression of most of the ion pumps came back to the normal levels within 6h of fresh water treatment. This suggests that osmoregulation is more efficient when louse is sitting on the fish and is able to take osmolytes form the host. Hence, the osmoregulatory balance is achieved faster.

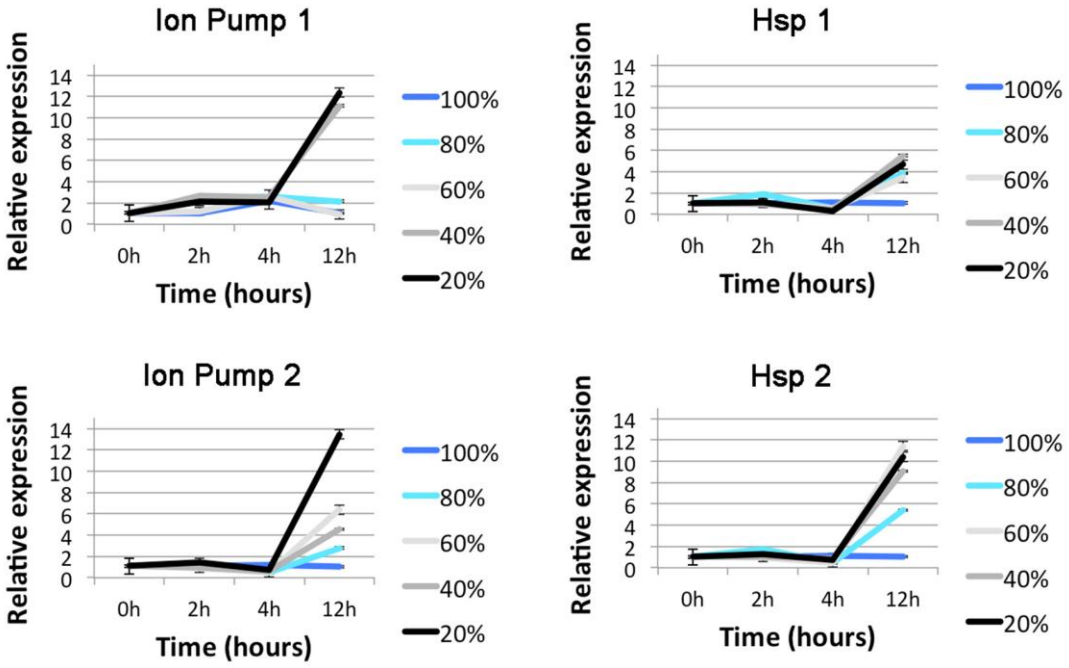

Figure 13. Changes in gene expression in copepodids after fresh water treatment.

RNAi experiments in copepodids.

RNA interference on nauplia I stage is a well-established method allowing evaluation of the gene function in salmon lice [12]. dsRNA knock-down the expression of the target gene and eliminate protein product of this gene. Potential phenotype is dependent on physiological function of the protein product.

In order to evaluate function of each ion pump, all genes were checked by RNA interference in nauplia I stage. Down-regulation efficiency was tested by qRT-PCR. Majority of tested genes were down-regulated in the range between 40 and 90%, which is an acceptable range for this type of experiment (Figure 14).

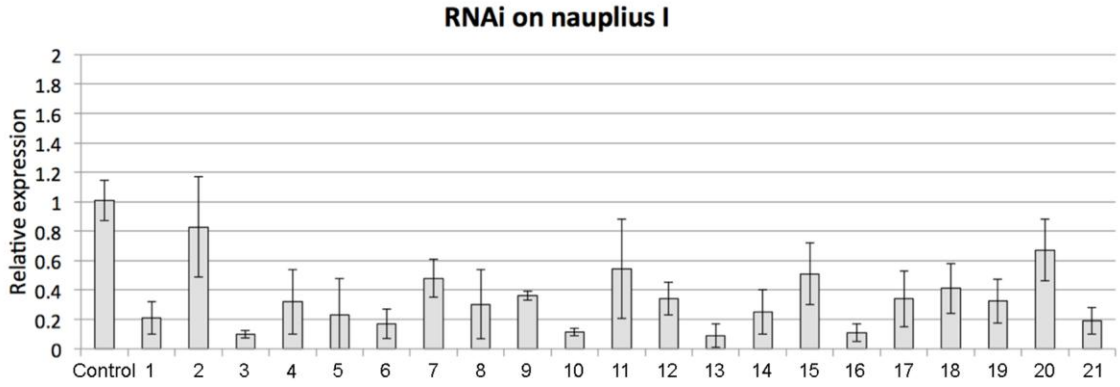

Figure 14. Gene expression analysis in knockdown lice and control animals. Transcript levels were quantified by qRT-PCR using RNA extracted from copepodids soaked with dsRNA against ion pump genes and cod trypsin CYP185 (control). Columns show mean expression levels and error bars show the standard deviation for each treatment group. N = 5 from each group.

All dsRNA treated copepodids were observed for potential phenotypes. Three genes showed significant functional and/or anatomical phenotype. Copepodids treated with dsRNA targeting gene 1 had abnormally developed gut with huge, inflated cells (Figure 15). Copepodids treated with dsRNA targeting gene 2, were significantly shorter and wider than controls (Figure 15). Copepodids treated with dsRNA targeting gene 3 did not show any visible anatomical abnormalities (data not shown). However, copepodids had restricted abilities for movement, which were progressively more severe with time, from normally behaving copepodids on day 5 post hatching to disability to swim on day 10.

Figure 15. Morphology of RNAi treated copepodids on day 7 post hatching. Anatomical abnormalities are visible in copepodids treated with dsRNA targeting gene 1 and gene 2. Gene 1 knock-down animals have enlarged, bloated cells in the intestine, while gene 2 knock-down animals are wider and shorter than controls.

RNA interference on pre-adult II females

To learn more about the functions of ion pumps and the process they are involved in, an RNAi approach was used to knock-down the expression of these genes in females during the maturation from pre-adult to adult. The knock-down of three tested genes was confirmed by qRT-PCR (Fig.16). Transcripts levels of all genes were significantly reduced when compared to the control group. Gene 1 transcript levels were reduced by 60% in gene 1 knockdown lice, Gene 2 transcript levels by 78% in Gene 2 treated animals, and Gene 3 transcript levels by 90% in knockdown lice.

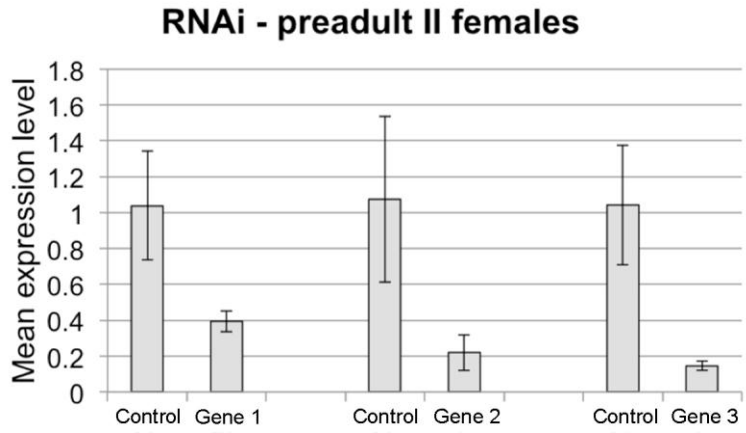

Figure 16. Gene expression analysis for Gene 1, 2 and 3 in knockdown lice and control lice. Transcript levels were quantified by qRT-PCR using RNA extracted from adult females injected with dsRNA against Gene 1, 2 and 3 and cod trypsin CYP185 (control). Columns show mean expression levels and error bars show the standard deviation for each treatment group. N = 5 from each group.

On the termination day for the RNAi experiment, all injected females were evaluated for anatomical phenotype. Gene 1 knock-down female had intestines full of blood and produced egg string (Figure 17). However, animals hatching form these egg strings were smaller than the controls and hatching success was reduced by around 40%. Gene 2 knock-down females did not produce egg strings and had reduced gut content (Figure 17). Similar phenotype was observed in lice with gene 3 knock-down (Figure 17).

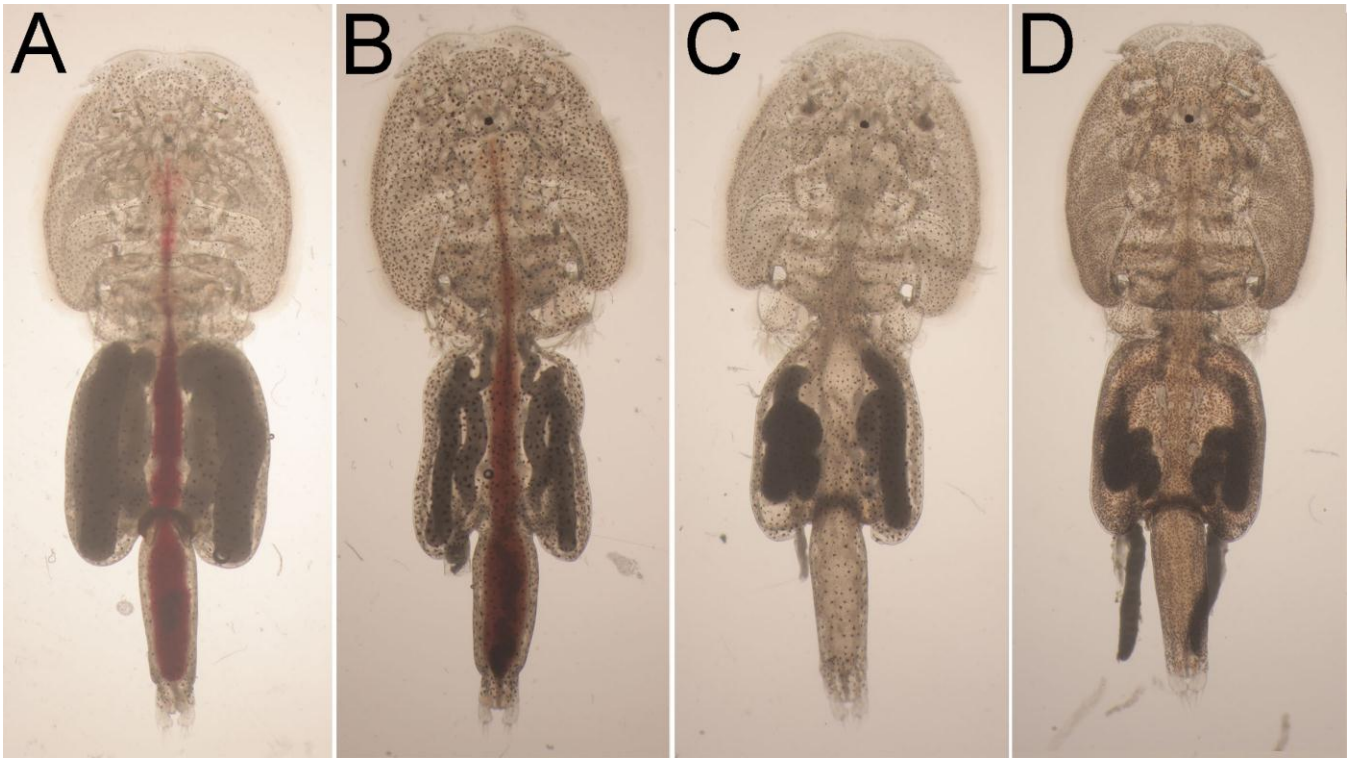

Figure 17. Morphology of RNAi treated animals at termination. (A) Normally developed control animal with filled genital segment (normal oocytes). (B) Gene 1 knockdown female. All animals produced egg strings and revealed normal development. But the hatching success was 40% lower then controls (C) Gene 2 knockdown. No animals produced egg strings, and reduced gut content could be observed in some individuals. Oocytes in the genital segment are not maturated properly. (D) Gene 3 knockdown. No animals produced egg strings, and reduced gut content could be observed in all individuals. Oocytes in the genital segment are not matured properly.

Histological examination revealed that females with gene 2 and gene 3 knock-down have severe morphological defects in the reproductive system (Figure 18), intestine and subcuticular tissue. Treated with dsRNA targeting gene 2 fail to develop eggs. Their oviducts are filled with disordered tissue (Figure 18). Females treated with dsRNA targeting gene 3 were able to produce mature eggs, however, they were abnormally shaped.

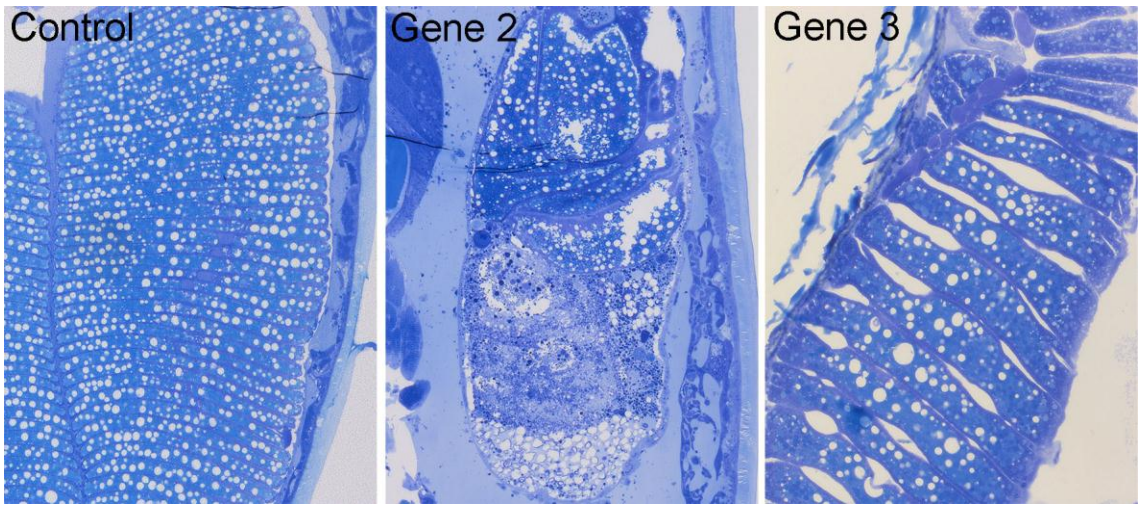

Figure 18. Histology of RNAi treated animals at termination. In control females' mature oocytes in the genital segment are properly formed and ready to be extruded. Gene 2 knockdown females fail to produce oocytes in any viable form. Gene 2 knockdown females produce egg, but they are thicker and wider compared to the control. In addition, empty spaces occurs between the oocytes in the genital segment.

In order to evaluate the expression patter of these genes, in situ hybridization was performed on sections of an adult female. In the adult female, both genes have a similar expression pattern, and are expressed in a number of the vital tissues and organs. Transcripts of both genes are localized to the ovary (Figure 18) and in maturing oocytes in the oviduct and in the membrane surrounding mature oocytes in the genital segment. Transcripts were also detected in the intestine (Figure 18) and subcuticular tissue.

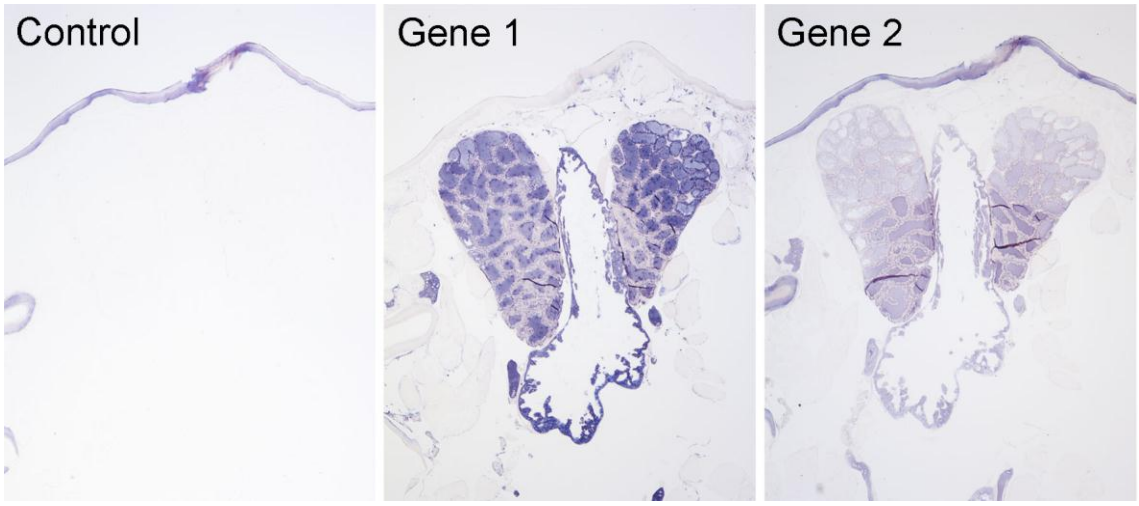

Figure 16. Expression of gene 2 and gene 3 in adult females. Transcripts were detected in reproductive system of adult female and in the intestine.

General conclusion Part 2:

In the light of constantly growing resistance against commercially used medicines, there is a high need to develop new means to control salmon louse. In order to fight salmon louse, it is crucial to gain detailed knowledge about the most crucial physiological processes for lice survival. One of them is ormoregulation and ability to maintain the stable ion balance of internal fluids. Salmon louse as an obligatory marine organism must be able to regulate and act to all negative changes taking place in the environment. Therefore, detailed knowledge about the main players in osmoregulation in salmon louse is necessary. Ion pumps are known to be involved in this process and they could be important target in the fight against lice. Moreover, because of high sequence variability between aquatic species, a number of these proteins could potentially be good targets for drug development or vaccine targets against salmon louse.

In this study, *L.salmonis* ion pumps were identified and analysed. Obtained results indicated that ion pumps regulate not only osmoregulation but are also involved in reproduction and feeding. Severe phenotype observed after knock-down of ion pumps, manifested by restricted ability to infect fish (lack of movement), feed (reduced feeding) and reproduce (abnormal or lack of eggs), greatly reduce animal's survival. Additionally, the fact that some of these genes are expressed in the intestine facilitates potential treatment.

6. Leveranser

Presentations:

- SLRC Workshop, Bergen. Osmoregulation in Salmon Lice. OSMO_LUS. 11-12.05.2016.
- SLRC Meeting, Bergen. Report of fresh water project. OSMO_LUS. 08.12.2016.
- SLRC Meeting, Bergen. Ion Pumps in salmon louse. OSMO_LUS. 15.03.2017.

Publications in progress:

- Andreas Borchel, Anna Zofia Komisarczuk, Alexander Rebl, Tom Goldammer, Frank Nilsen. Systematic identification and characterization of stress-inducible heat shock proteins (HSPs) in the salmon louse (Lepeophtheirus salmonis) (2017). Manuscript in progress.
- Three manuscripts are in progress, describing ion pumps influence on physiology and development of salmon louse (Lepeophtheirus salmonis) – manuscript in progress;

1. Hahnenkamp, L. and H.J. Fyhn, *The Osmotic Response of Salmon Louse, Lepeophtheirus-Salmonis (Copepoda, Caligidae), during the Transition from Sea-Water to Fresh-Water.* Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology, 1985. **155**(3): p. 357-365.
2. Guo, H., et al., *Trascriptome analysis of the Pacific white shrimp Litopenaeus vannamei exposed to nitrite by RNA-seq.* Fish & Shellfish Immunology, 2013. **35**(6): p. 2008-2016.
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5. Lv, J.J., et al., *Transcriptome Analysis of Portunus trituberculatus in Response to Salinity Stress Provides Insights into the Molecular Basis of Osmoregulation.* Plos One, 2013. **8**(12).
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11. Dalvin, S., F. Nilsen, and R. Skern-Mauritzen, *Localization and transcription patterns of LsVasa, a molecular marker of germ cells in Lepeophtheirus salmonis (KrOyer).* Journal of Natural History, 2013. **47**(5-12): p. 889-900.
12. Eichner, C., et al., *A method for stable gene knock-down by RNA interference in larvae of the salmon louse (Lepeophtheirus salmonis).* Experimental Parasitology, 2014. **140**: p. 44-51.