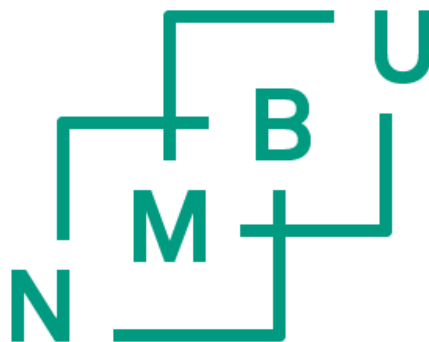


Monitoring of drug resistance and resistance development mechanisms in salmon lice (*Lepeophtheirus salmonis*)

Kari Olli Helgesen

Thesis for the degree of *Philosophiae Doctor*



Department of Food Safety and Infection Biology

Faculty of Veterinary Medicine and Biosciences

Norwegian University of Life Sciences

Adamstuen, 2015



Thesis number 2015:44

ISSN 1894-6402

ISBN 978-82-575-1957-5

TABLE OF CONTENTS

Acknowledgements	4
List of papers	5
Acronyms/terminology	6
Summary	7
Sammendrag	8
Introduction	9
Salmon farming	9
Diseases in farmed salmonids	10
Salmon lice	11
Treatments of salmon lice	13
Drug resistance	15
Resistance testing and resistance mechanisms	18
Aims of the study	21
Methods	22
Main results	27
Discussion	32
Aim 1	32
Aim 2	35
Aim 3	37
Aim 4	39
Overall discussion	41
Conclusion	42
Future perspectives	42
References	45
Enclosed papers	58

ACKNOWLEDGEMENTS

The work included in this thesis was performed at the Norwegian University of Life Sciences (NMBU), formerly the Norwegian School of Veterinary Science, between the years 2011 and 2015. The studies were financed by the the Norwegian Research Council with additional funding from The Norwegian Seafood Research Fund for PrevenT, under the platforms PrevenT (NFR 199778/S40) and the Sea Lice Research Centre (SLRC) (NFR 203513/030).

The studies would not have been possible without the goodwill of several fish farming companies who allowed us to sample their fish for salmon lice. The field sampling was performed with the help of the companies Helgeland Havbruksstasjon AS, Aqua kompetanse AS, Havbrukstjenesten AS and VESO. Aqua kompetanse AS and employee Harriet Romstad deserves special thanks for performing the field hydrogen peroxide bioassays included in paper II. The staff working at NIVA Marine Research Station also deserves recognition for looking after our fish when we were not there.

I would like to thank my co-authors for their contribution to the papers included in this thesis. Frank Nilsen and Heidi Kongshaug at the SLRC at the University of Bergen deserve thanks for laying a foundation stone in the work on paper IV. The reviewers of the different papers also deserve thanks for improving our work by taking their time to critically evaluate the manuscripts.

My supervisors must be thanked especially: My main supervisor Tor Einar Horsberg who taught me all I know about being a researcher and who managed to pour oil on troubled waters when I was at my most frustrated, my co-supervisor Kiranpreet Kaur who led me into the world of molecular biology and my co-supervisor Henning Sørum who always took his time answering my questions.

I am grateful to my colleagues in the lice-group at NMBU: Marit Bakke, Kiranpreet Kaur, Stian Mørch Aaen, Melanie Andrews and Celia Ridaura for creating an excellent working environment. It has been great having the group; to learn from and to cooperate and discuss with. Together with the rest of the section for Pharmacology and Toxicology the group also creates a social working environment to thrive in. Janicke Nordgreen, Josephine Prendergast and Rune Landsem must be emphasized especially for helping me with statistics, linguistics and chemical analysis, respectively.

Outside of my closest colleagues, but still in the same situation as me: I would like to thank Ingrid Holmøy and Runa Rørtveit for sharing their PhD-frustrations with me and listening to mine. Together we managed to reduce their importance.

I would also like to thank my family and friends who have stood by me and supported me through these years. My four legged friend also deserves thanks for being my personal trainer, my everyday companion and a constant source of joy. And thanks to my dearest Asbjørn for giving my life an extra dimension.

LIST OF PAPERS

Paper I

Helgesen, K.O., Horsberg, T.E., 2013. Single-dose field bioassay for sensitivity testing in sea lice, *Lepeophtheirus salmonis*: development of a rapid diagnostic tool. *Journal of Fish Diseases* 36, 261-272.

Paper II

Helgesen, K.O., Romstad, H., Aaen, S.M., Horsberg, T.E., 2015. First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway. *Aquaculture Reports*, doi:10.1016/j.aqrep.2015.01.001.

Paper III

Kaur, K., Helgesen, K.O., Bakke, M.J., Horsberg, T.E., 2015. Mechanism behind resistance against the organophosphate azamethiphos in salmon lice (*Lepeophtheirus salmonis*). *PLOS ONE*, in press.

Paper IV

Helgesen, K.O., Nilsen, F., Kongshaug, H., Kaur, K., Horsberg, T.E., 2015. Voltage gated sodium channel genes in the salmon louse (*Lepeophtheirus salmonis*): Gene characterizations and sequence analysis of knockdown resistance-homologous regions in pyrethroid resistant parasites. (Manuscript).

ACRONYMS/TERMINOLOGY

AChE	Acetylcholinesterase. Enzyme hydrolyzing acetylcholine
AGD	Amoebic gill disease. A parasitic disease
Bioassay	Biological assay. Applied as resistance tests
CDC	Centers for Disease Control and Prevention. Public health agency in USA
cDNA	Complementary DNA. Double-stranded DNA synthesized from RNA
EMB	Emamectin benzoate. Anti-salmon lice agent
FAO	The Food and Agriculture Organization of the United Nations
Field resistance	Reduced treatment efficacy due to resistance
Fitness cost	Reduced ability to survive and reproduce due to being resistant
HRM	High resolution melt. Laboratory analysis to detect a mutation
H ₂ O ₂	Hydrogen peroxide. Anti-salmon lice agent
Instar	General term for a developmental stage of salmon lice
ISA	Infectious salmon anemia. A salmon viral disease
Louse strain	Lice originating from a specific salmon farm collected at a specific time
NIVA	Norwegian Institute for Water Research
NOK	Norwegian kroner. The Norwegian currency
PCR	Polymerase chain reaction. To amplify a specific part of DNA
qPCR	Quantitative PCR. To measure the expression level of a gene
RACE PCR	Rapid amplification of cDNA ends. To obtain full length sequences of RNA
Resistance	Genetically based decrease in susceptibility to a pesticide
Salmon louse	<i>Lepeophtheirus salmonis</i> . Abbreviated Ls in the names of the louse strains
Salmonid	Fish belonging to the family salmonidae
Sea louse	Marine ectoparasites in the family Caligidae, subphylum Crustacea
SNP	Single nucleotide polymorphism. DNA variations in a single nucleotide.
TaqMan	Probes designed to increase the specificity of quantitative PCR
WHO	World Health Organization

SUMMARY

The parasite salmon louse (*Lepeophtheirus salmonis*) poses a threat to the salmonid aquaculture in the Northern Hemisphere. Depending on the developmental stage and the abundance of lice, these parasites can cause stress, skin erosions, wounds, anaemia and osmoregulatory difficulties in the fish. A large infestation may even be fatal to their hosts. The direct effects of the lice can create gateways for secondary infections. Furthermore the parasites may also act as passive vectors for other disease agents. Due to their huge reproductive capacity and the presence of planktonic life stages, the salmon lice from one fish farm can infest other fish at the same farm, in neighbouring farms as well as wild salmonids. To reduce the infestation pressure on wild salmonids and the potential harmful effects on the farmed fish, it is necessary to constantly maintain a low level of salmon lice on the fish. This has predominantly been achieved by the use of chemical treatments.

Due to both frequently applied treatments and dependence on a limited number of chemical treatment agents, salmon lice have developed resistance towards the most commonly used chemotherapeutants. When a chemical treatment fails, it is necessary to determine if the failure was due to resistant salmon lice or an erroneously performed treatment. This is achieved through the use of resistance tests which are also useful in determining the pre-treatment sensitivity level of the salmon lice. This information helps avoid carrying out unsuccessful treatments, which are expensive for the fish farmer as well as stressful and potentially harmful for the fish and the marine environment. The biological assays (bioassays), which are traditionally applied as resistance tests, require many parasites, delicate handling of the lice and are performed using various protocols. The aim of the current thesis was to refine existing methods and develop new techniques for the monitoring of drug resistance in salmon lice.

In paper I and II, a new bioassay protocol using standardised equipment was developed, with 24 hours exposure to all treatment chemicals. A simplified method was used to evaluate the results. The protocol was developed for the following treatment chemicals; deltamethrin, azamethiphos, emamectin benzoate and hydrogen peroxide. These assays were able to differentiate between resistant and sensitive strains of salmon lice, in accordance with results from the traditional bioassays and small-scale treatments. Furthermore in paper II, a bioassay protocol with 30 minutes exposure to hydrogen peroxide was developed and hydrogen peroxide resistance was described for the first time in salmon lice from Norway.

Paper III and IV addressed the molecular mechanisms of organophosphate and pyrethroid resistance in salmon lice. This was achieved by screening for mutations in the target sites acetylcholinesterase (AChE) and voltage gated sodium channels (Na_v). A mutation was detected in one of the two AChE genes and its association with organophosphate resistance proven. The frequency of the mutation varied according to the sensitivity of the salmon louse strains. 3D modelling showed the effect of the mutation in the protein while enzymatic assays showed a reduced inhibition of the enzyme by organophosphates, in resistant when compared to sensitive parasites. A rapid laboratory based resistance assay was subsequently developed in order to detect the mutation. The three Na_v genes in salmon lice were identified and characterized in paper IV. Differences in expression of any of the three genes were not shown as a cause of resistance. No mutations were detected during screening of the most highly expressed Na_v gene. This applied to resistant parasites from different parts of Norway. A rapid resistance assay could therefore not be developed to detect pyrethroid resistance.

SAMMENDRAG

Lakselus (*Lepeophtheirus salmonis*) er en parasitt som utgjør en stor trussel mot oppdrett av laksefisk på hele den nordlige halvkule. Infeksjoner med lakselus stresser fisken. I tillegg kan lusene forårsake huderosjoner, sår og anemi, og gi fisken problemer med osmoreguleringen. En kraftig infeksjon kan være dødelig for fisken. De direkte effektene av lus kan skape inngangsporter for sekundærinfeksjoner. Lus kan også fungere som mekaniske vektorer for andre sykdomsagens. På grunn av den store reproduksjonskapasiteten til lus, samt at flere av livsstadier er planktoniske, utgjør lus fra ett anlegg en smitterisiko for andre fisk på samme anlegg, for fisk på naboanlegg og for vill laksefisk. For å begrense smittefaren til villfisk og for å redusere risikoen for sykdom på fisk i eget anlegg, må oppdrettere holde lusenivået nede. Kjemisk avlusing av fisk har vært den absolutt vanligste måten for å holde kontroll på lusetallet.

Tilgang til få ulike kjemiske avlusningsmidler samt hyppige behandlinger har ført til at lakselus har utviklet resistens mot behandlingsmidlene. Dersom resultatet fra en behandling ikke blir som forventet er det nødvendig å ha resistenstester, slik at en kan finne ut om behandlingssvikten var forårsaket av resistente lus eller av feilaktig utført behandling. I tillegg er det nyttig å utføre resistenstester før behandling, slik at en unngår mislykkede behandlinger. Kjemisk avlusing er kostbart for oppdretteren og stressende og potensielt skadelig for fisken og miljøet. De tradisjonelle resistenstestene krever mange lus, en skånsom håndtering av lusene, og blir utført etter flere ulike protokoller. Målet med denne avhandlingen var å forbedre og forenkle de allerede eksisterende resistenstestene samt å utvikle nye teknikker for å resistensteste lus.

I artikkel I og II ble det utviklet et nytt biologisk assay (bioassay) for resistenstesting av lakselus, med 24 timers eksponering for lusemidlene deltametrin, azametifos, emamektin benzoat og hydrogen peroksid (H_2O_2). Utstyret som ble brukt til assayet ble standardisert og avlesningen av resultatene ble forenklet. Dette assayet skilte mellom sensitive og resistente lus på samme måte som det tradisjonelle assayet og små-skala behandlingsforsøk gjorde. I artikkel II ble det i tillegg utviklet et H_2O_2 bioassay med 30 minutters eksponering for kjemikalet. Dette studiet var det første som påviste H_2O_2 -resistens hos lakselus i Norge.

I artikkel III og IV ble det lett etter den molekylærbiologiske mekanismen bak organofosfat- og pyretroidresistens ved å screene etter mutasjoner i disse kjemikalienes mål-steder i lakselus; henholdsvis acetylkolinesterase (AChE) og spenningsstyrte natriumkanaler (Na_v). I en av AChE genene ble det funnet en mutasjon som var sterkt assosiert med azametifosresistens. Frekvensen av mutasjonen varierte i takt med lusestammens sensitivetsnivå, 3D-modellering av proteinet viste at mutasjonen endret proteinets egenskaper og enzymkinetikk-undersøkelser viste at enzymet ble hemmet av organofosfater i mindre grad i resistente enn i sensitive lus. Det ble derfor utviklet en rask laboratorietest for å detektere mutasjonen. I artikkel IV ble de tre ulike Na_v genene til lakselus beskrevet. Det ble utført en studie på genuttrykket av de tre genene i resistente og sensitive lus, uten at det ble funnet noen forskjeller. I tillegg ble det mest uttrykte genet screenet for mutasjoner i resistente lus fra ulike lokaliteter i Norge, uten at noen mutasjoner ble oppdaget. Det kunne derfor ikke utvikles en rask laboratorietest for pyretroidresistens i lakselus.

INTRODUCTION

Salmon farming

Marine aquaculture of salmonid (family: salmonidae) fish species began in the late 50s/early 60s in Norway when smolts of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) were placed in fish cages at sea. Caging the fish enabled farmers to exploit these species' vast growth potential in order to produce valuable and popular fish meat from lower priced food sources. From that point in time, the salmon farming industry has grown substantially and is today a major contributor to food production, employment and the Norwegian export economy. In 2013 3.5 % (31.6 billion NOK) of the total Norwegian export income was from Atlantic salmon (Statistics Norway, 2014). In addition to Norway, the main contributors to marine salmonid farming are Chile, Scotland, Canada, the Faroes, US, Australia and Ireland (Food and Agriculture Organization of the United Nations, 2015). The annual world production of farmed Atlantic salmon between 1980 and 2012 is presented in Figure 1.

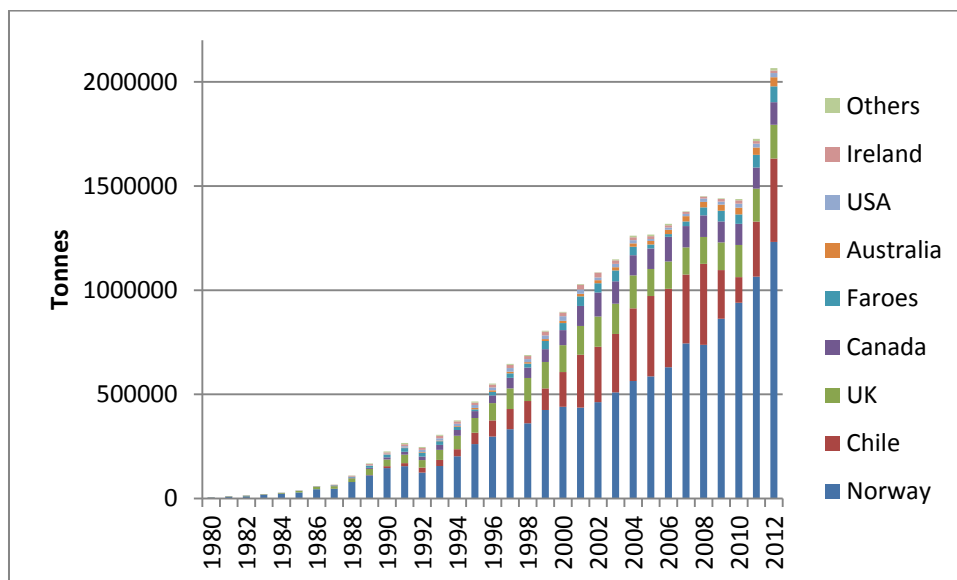


Figure 1. The figure presents the annual production of Atlantic salmon in tonnes between the years 1980 and 2012. The data are collected from the website of Food and Agriculture Organization of the United Nations (<http://www.fao.org/fishery/statistics/global-aquaculture-production/en>)

The majority of salmon farms are open net cage farms where the fish in the nets are in constant contact with their surrounding environment. Water is exchanged via natural currents and food spill and waste products are released to the surrounding area. Pathogens, including viruses, bacteria and parasites, can move freely between the cages within the fish farm, between neighbouring farms as well as between wild and farmed fish, by transfer in sea water. This is in stark contrast to smolt production, which primarily occurs in enclosed indoor facilities. According to the Food and Agriculture Organization of the United Nations (FAO), the most important species in marine salmonid farming today are Atlantic salmon, rainbow trout and coho salmon (*Oncorhynchus kisutch*) (Food and Agriculture Organization of the United Nations, 2015). The distribution of the world marine production of different salmonid species from 2012 is shown in Figure 2.

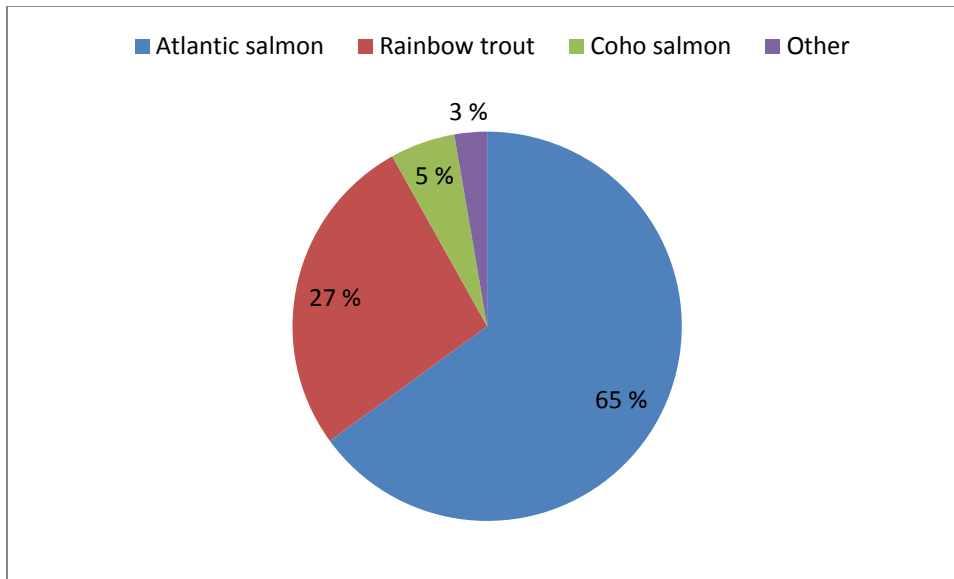


Figure 2. Distribution of the world marine aquaculture production of salmonids in 2012 according to the Food and Agriculture Organization of the United Nations (<http://www.fao.org/fishery/statistics/global-aquaculture-production/en>). The production in tonnes was: Atlantic salmon: 2 066 561, rainbow trout: 855 982, Coho salmon: 171 681, other: 86 136.

Despite being economically viable, marine salmonid farming faces several challenges with respect to environmental pollution, fish diseases and animal welfare issues. Both organic contents from food spills and waste products from the fish contribute to the build-up of organic matter beneath the farms (Pohle et al., 2001). Furthermore the occurrence of changes in the species diversity has also been observed in the nearby areas (Kutti et al., 2007). The chemicals which are applied to combat diseases may also influence wild living organisms (Ernst et al., 2001; Langford et al., 2014). Finally escaped fish may contribute to genetic pollution of local wild fish stocks if they migrate into rivers and spawn (Mork, 1991).

The unnatural environment, in which the farmed fish are kept in, leads to challenges regarding animal welfare. In comparison to wild fish, there is a greater density of farmed fish, which subsequently can result in a greater exposure of the latter group to disease agents. The net caging deprives the farmed fish of the possibility to perform several of salmonid's natural behaviours. Fish are also stressed from the different handling procedures they are put through during a production circle. This involves disease treatments and transport and may be extra harmful if the procedures are carried out sub-optimally (Bowers et al., 2002; Iversen et al., 2005).

Diseases in farmed salmonids

Disease in farmed salmonids leads to health and welfare issues for the affected fish. An outbreak of a disease may also have more far-reaching consequences as many diseases in salmonid aquaculture are contagious. The diseased fish may infect other fish at the same farm as well as in neighbouring farms. Furthermore the disease may spread to wild salmonids. Due to the development of vaccines, the outbreaks of bacterial diseases have greatly diminished. This is reflected in the reduction in antibiotic usage in Norwegian aquaculture, despite the huge increase in the production of salmonids in the same time period (Midtlyng et al., 2011).

Vaccines have good preventive effects against the following bacterial diseases; vibriosis caused by *Vibrio (Listonella) anguillarum*, cold water vibriosis caused by *Vibrio salmonicida*, furunculosis caused by *Aeromonas salmonicida* and enteric redmouth disease caused by *Yersinia ruckeri* (Gudding et al., 2010; Tobbäck et al., 2007; Toranzo et al., 2005). There are vaccines which partly prevent winter ulcers caused by *Moritella viscosa*, as well as vaccines which partly prevent the intracellular bacterial diseases bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* and the salmon rickettsial disease caused by *Piscirickettsia salmonis* (Gudding et al., 2010; Toranzo et al., 2005). The latter disease is considered the most imperative with respect to Chilean salmonid farming (reviewed in Rozas and Enriquez, 2014).

The most prevalent viral diseases include infectious salmon anaemia (ISA) caused by a virus in orthomyxoviridae (Falk et al., 1997), pancreas disease caused by salmonid alphavirus (Weston et al., 1999), infectious pancreas necrosis caused by a virus in birnaviridae (Wolf, 1988), heart- and skeletal muscle inflammation caused by piscine reovirus (Palacios et al., 2010) and cardiomyopathy syndrome caused by a virus in totiviridae (Lovoll et al., 2010). In the Pacific Ocean in US and Canada, infectious haematopoietic necrosis virus (IHNV) causes disease in salmonids (Saksida, 2006).

Tapeworm from the genus *Eubothrium* are noteworthy internal parasites in salmonid aquaculture, while the amoebic gill disease (AGD) caused by *Neoparamoeba perurans* and sea lice (family Caligidae, subphylum Crustacea) infestation, mainly by *Lepeophtheirus salmonis* or *Caligus rogercresseyi*, are important external parasitic diseases (Bristow and Berland, 1991; Ruane and Jones, 2013; Torrissen et al., 2013; Young et al., 2007). The former disease is the most pertinent disease which is currently threatening the Australian (Tasmanian) salmonid farming. However this disease is also emerging in other areas (Ruane and Jones, 2013). Sea lice are causing widespread concern throughout most of the salmonid producing world.

Salmon lice

The salmon louse *L. salmonis* is a parasitic arthropod with a direct life cycle, belonging to the subphylum crustacea, subclass copepoda. The life cycle of *L. salmonis* is composed of 8 stages; two planktonic nauplius stages, one infective copepodite stage, two chalimus stages where the parasite is attached to the host, two pre-adult stages and one adult stage. In the three latter stages, the parasite moves freely around on its host. Between each stage is a moult, where the parasite changes its exoskeleton. Growth is also evident within an instar. This, combined with the size difference between the male and female salmon louse, is the reason behind the recent discovery that *L. salmonis* has two and not four chalimus stages (Eichner et al., 2015; Hamre et al., 2013). At 10 °C the parasite's life cycle takes about six weeks. However the length of the cycle is dependent on the temperature of the water (Wootton et al., 1982). The parasites' fecundity is large and in the laboratory a single female has been seen to produce 11 pairs of egg strings during its lifetime. In the same experiment the mean number of viable eggs per string was 152 (Heuch et al., 2000).

L. salmonis is a parasite of salmonid fish in the Northern Hemisphere and the species is divided into the two subspecies *L. salmonis salmonis* in the Atlantic Ocean and *L. salmonis oncorhynchi* in the Pacific Ocean (Skern-Mauritzen et al., 2014). Atlantic salmon, trout (*Salmo trutta*), chum salmon (*Oncorhynchus keta*) and rainbow trout are highly susceptible hosts. Other Pacific salmonids may also act as hosts, but they are less prone to salmon lice infestations (Johnson and Albright, 1992; Sutherland et al., 2014a). The parasitic stages feed on the mucus, skin and blood of their hosts

(Brandal et al., 1976; Kabata, 1974). The combination of the great abundance of suitable hosts in fish farms, the major reproductive capacity of the parasite and their effect on the host, explains why *L. salmonis* is considered a major problem in the salmonid farming world.

In Chile in the Southern Hemisphere the parasite, which is causing the greatest concern in the fish farming industry, is the sea louse *C. rogercresseyi*. Despite the fact that this parasite is smaller than *L. salmonis*; due to its abundance the pathologic potential is large. Huge efforts are therefore made to control *C. rogercresseyi* in Chile (Bravo, 2003). Caligus species in the Atlantic Ocean, predominately *Caligus elongatus*, also infest salmonids in the fish farms (reviewed in Boxaspen, 2006). At the current point in time, sea lice are not considered a major salmonid health issue in Australia (Nowak et al., 2011).

L. salmonis pose both a health and a welfare threat to the farmed salmonids. By feeding on the skin and blood of their hosts the parasites can induce wounds as well as cause anaemia (Grimnes and Jakobsen, 1996; Jonsdottir et al., 1992). Wounds are problematic given that they are painful. Furthermore they may act as gateways for secondary infections as well as lead to osmoregulatory difficulties. The latter is due to the fact that fish need their skin intact in order to maintain homeostasis in their salt-balance (Grimnes and Jakobsen, 1996). The damage to the fish might even be fatal when heavily infested (Grimnes and Jakobsen, 1996). Increased risk of other infections may also arise as a consequence of lice-induced immune suppression. In order to prevent attacks from the hosts' immune system, salmon lice trigger immune suppression at the attachment sites to their hosts (Braden et al., 2012). They also provoke a stress response, seen as changes in serum electrolyte and hormone levels, in the fish (Bowers et al., 2000; Nolan et al., 1999). Furthermore the treatments of salmon lice may be stressful for the fish (Bowers et al., 2002). Simultaneous suppression of antiviral immunity and increased salmon lice infestation has been observed in Pacific salmon (Sutherland et al., 2014a). Given that a higher frequency of treatments of salmon lice have been shown to reduce the risk of ISA infections in Atlantic salmon (McClure et al., 2005); it is reasonable to assume that salmon lice infestation increase the risk of this disease. *L. salmonis* has also been shown to be a possible mechanical vector for the infectious hematopoietic necrosis virus and the infectious salmon anaemia virus (Jakob et al., 2011; Nylund et al., 1993).

Salmon lice are a threat to the wild salmonids, especially to the migrating smolt and to the local sea trout populations in fjords. Given that many fish farms are located near migrating rivers and that farmed salmonids far outnumber wild salmonids in the Atlantic Ocean; copepodites which infest wild salmonids most likely arise from fish farms. Undoubtedly salmon lice have a negative effect on the infested individuals, but whether or not they have a detrimental effect on wild salmonid population is still deliberated. Evidence from Pacific Canada supports the theory that salmon lice can have a fatal effect on wild salmonids (Krkošek et al., 2007), while Riddell et al. (2008) claimed that Krkošek et al. (2007) overestimated the effect of salmon lice on the decline of pink salmon. Jackson et al. (2013) found minor regulatory effects of salmon lice on the Atlantic salmon population in Ireland. However this conclusion was also questioned by others (Krkošek et al., 2014).

The economic costs connected to salmon lice are both a direct result of their effects on the host as well as a result of the measures taken to combat the parasites. According to their abundance on the fish, the lice can damage the fish skin and thereby possibly result in degrading of the fish at slaughter. Secondary infections in the lice-induced wounds as well as infections following immune

suppression triggered by the parasites, may lead to a substantial loss of fish. Treating fish for salmon lice is expensive, as is all non-chemical measures implemented to combat the parasites. The chemicals are costly due to the large quantities which are required for the treatments of salmon lice and the labour- and machinery costs, which increase drastically when performing a treatment.

Costello (2009) estimated treatments costs alone to make up 6 % of the value of salmonid production. In 2012 the FAO estimated the value of the world production of Atlantic salmon and rainbow trout to be more than 13 billion USD (Food and Agriculture Organization of the United Nations, 2015). According to Costello's estimation this puts sea lice treatments at a cost of more than 700 million USD for these aforementioned fish species. Due to the fact that treatments involves days of starvation, potential weight gain of the fish is also lost. With respect to the authorities, salmon lice are also expensive parasites, as it becomes necessary to employ people in both the regulatory and supervisory authorities as well as at research institutions. This is needed in order to deal with various aspects of the parasitic disease. As long as full control is not gained, one of the greatest impacts the salmon lice have on the economy is the limitations they set for future growth of salmonid production. If the salmon farming industry has difficulties controlling today's amount of salmon lice, regulatory authorities will be reluctant in allowing them to increase their production.

Treatments of salmon lice

In order to combat salmon lice infestations several possible measures can be implemented. Traditionally chemical treatment of salmon lice has been the most applied method. Chemical treatments may be given as bath treatments in enclosed tarpaulins, in well boats, or as in-feed treatments (reviewed in Grant, 2002). Bath treatments require crowding of the fish. Furthermore with respect to well boat treatments, the fish have to be transferred back and forth from the cage to the boat. The chemicals, currently applied in anti-salmon lice bath treatments, are the pyrethroids deltamethrin and cypermethrin, the organophosphate azamethiphos and hydrogen peroxide (H₂O₂). The avermectin emamectin benzoate (EMB) and the benzoylureas deflubenzuron and teflubenzuron comprise the chemicals which are applied in in-feed treatments (Roth, 2000). Due to regulatory differences, the availability of the various pesticides varies between countries and regions (reviewed in Aaen et al., 2015).

Each treatment of salmon lice places the fish at risk for injuries induces by crowding or by contact with technical installations. The treatments also increase the risk of death by hypoxemia due to the fact that the water circulation is stopped during a bath treatment (Treasurer et al., 2000a). Intoxication as a result of an overdose of the treatment agent also endangers the fish (Haya, 1989; Thomassen, 1993). Additional oxygen is supplied during bath treatments, but this system may fail to provide adequate amounts. Intoxication may occur due to the fact that some of the chemicals have limited safety margins. Furthermore in an attempt to combat resistant parasites, increased doses may be applied. Products may also be applied at temperatures where they become more toxic for the fish. In a study on hydrogen peroxide, no mortality in Atlantic salmon was seen at 10 °C, whereas the same concentration at 13.5 °C killed 35 % of the treated fish (Bruno and Raynard, 1994).

Chemical treatments of salmon lice result in the discharge of chemicals to the surrounding environment of the farms, subsequently placing other wild living organisms at potential risk. This has been shown for wild crustaceans such as shrimp, crab and lobster (Burrige et al., 2014; Samuelson et al., 2014). In samples collected from Norwegian fish farms in 2008 and 2010, potentially dangerous

levels of teflubenzuron to shrimps were detected in this species (Langford et al., 2014). Negative media attention arises both from the danger the salmon lice, originating from sea farms, pose to wild salmonids as well as the negative effects of medicinal discharge on the surrounding environment. This in turn has negative consequences for the public perception of salmon farming.

The modes of action of the chemical treatment agents in salmon lice have not been studied in this species directly, but they are believed to act in a similar manner in salmon lice as in other species. Pyrethroids act by altering both the activation and the inactivation of the voltage gated sodium permeable channels in nerve cell membranes of arthropods. As a result the normal neural impulse is discontinued, which leads to paralysis and eventual death of the exposed arthropod (reviewed in Bloomquist, 1996). Pyrethroids have an effect on all parasitic instars of salmon lice (Hart et al., 1997). Organophosphates inhibit acetylcholinesterase (AChE) in the post synaptic nerve cell membranes in cholinergic synapses. The neural transmitter acetylcholine is therefore not cleaved to acetate and choline and the neural impulse is prolonged. This results in paralysis and death of the parasites (reviewed in Fukuto, 1990). Azamethiphos only affects the mobile stages of salmon lice (Roth et al., 1996). The mode of action of hydrogen peroxide is unknown, but the substance is believed to cause cell damage due to its oxidative properties. H_2O_2 has for example been shown to oxidate amino acids (Finnegan et al., 2010). Gas bubbles in the gut and hemolymph, presumably oxygen from degradation of H_2O_2 , have been observed in some of the immobilized salmon lice (Bruno and Raynard, 1994). However the effect of H_2O_2 on the salmon lice is at least partly reversible as some of the parasites recover following immobilization (Bruno and Raynard, 1994; Hodneland et al., 1993; Treasurer and Grant, 1997). H_2O_2 predominately affects pre-adult and adult lice (Johnson et al., 1993; Thomassen, 1993). The chemical also hinders the hatching of parasite eggs as well as development into infective copepodites (Aaen et al., 2014; Johnson et al., 1993; Toovey and Lyndon, 2000). Emamectin benzoate acts on the γ -aminobutyric acid (GABA)- and glutamate gated chloride channels in nerve cells by maintaining the channels in an open state. This allows a constant flow of chloride into the cell and the normal neural impulse is disrupted. The parasite is therefore irreversibly paralysed (Arena et al., 1995; Duce et al., 1995). Electrophysiological evidences have been given for the effect of EMB on glutamate gated chloride channels in *C. rogercresseyi* (Cornejo, 2014). EMB acts on all parasitic instars of *L. salmonis* (Stone et al., 1999). Both diflubenzuron and teflubenzuron inhibit chitin synthesis subsequently preventing the parasites from moulting. This is due to the fact that chitin is a vital part of their exoskeleton (reviewed in Merzendorfer, 2013). These substances therefore have an effect on all parasitic instars of *L. salmonis*, except the adult stage (Branson et al., 2000).

Due to the factors such as chemical costs, environmental side-effects of chemical spill, potential hazards for the fish and operating personnel during treatments and resistance development in salmon lice; several non-medicinal lice control alternatives have been developed. These include co-stocking the salmon net cages with cleaner fish, a method which has been used for several years in order to provide a constant reduction of salmon lice. The species ballan wrasse (*Labrus bergylta*), goldsinny wrasse (*Ctenolabrus rupestris*), corkscrew wrasse (*Symphodus melops*), rockcook wrasse (*Centrolabrus exoletus*), cuckoo wrasse (*Labrus mixtus*) and lumpsucker (*Cyclopterus lumpus*) are the most commonly used cleaner fish (Costello, 1996; Imsland et al., 2014; Leclercq et al., 2014).

Several new technologies combatting salmon lice are either in use or are in a development phase. These technologies include the use of planktonic nets as skirts around the cages in order to hinder the vast majority of the salmon lice larvae from reaching the fish. This method is based on the fact

that larvae are predominately situated in the upper parts of the sea (Grøntvedt and Kristoffersen, 2015; Heuch et al., 1995). A snorkel cage has also been tested to force the fish to remain at deeper sea levels while they are simultaneously allowed access to open air to fill their swim bladders (Hanssen and Lie, 2014). Optic delousing, with laser situated in the net cages, is another technique which is presently in use in some salmon farms (Ramsden, 2014). Selection of the most lice resistant fish families by salmon breeding and the development of new anti-salmon louse feed ingredients are examples of other measurements which possibly can be taken (Glover et al., 2005; Hastie et al., 2013; Purcell et al., 2013). Treatments in well boats or fleets with warm sea water, fresh water, as well as mechanical delousing using high pressure sea water, are being used at smaller scale (Anonym, 2014; Berge, 2015; Wagner et al., 2004). The development of closed containers for fish farming (Tal et al., 2009) and anti-salmon lice vaccines are two areas of intense research. The former is currently still an expensive alternative to open net cages while the latter has proven to be an extremely difficult research task. Vaccines against *L. salmonis* have been developed, but show limited effects (Frost et al., 2007; Ross et al., 2006).

An integrated pest management (IPM) strategy, involving the application of all available tools against salmon lice, includes both chemical and non-chemical measures. IPM also includes operational decisions such as single year-class stocking, stocking with less susceptible species and area fallowing. In order to avoid the development of salmon lice resistance and thereby prolong the effect of the chemical treatment agents; reduction in the number of treatments performed, synchronized treatments in an area and rotation between substances with different modes of action are important. Furthermore surveillance of salmon lice levels at every farm is necessary in order to evaluate the actions implemented against the lice and thereafter decide which course of action to take (Brooks, 2009). Surveillance of salmon lice sensitivity to the different chemicals is an important factor in order to determine resistance and thereby predicting treatment efficacies to avoid inefficient treatments (Denholm et al., 2002). When information is shared between farmers in the same area and common decisions are made in an area regarding anti salmon lice measures, IPM becomes more efficient (Mordue and Pike, 2002).

Providing refugia for sensitive parasites in designated areas of farmland is a well-known strategy from resistance management in crop pests (Kruger et al., 2009). Refugia are also applied as a method for delaying resistance development of internal parasites in farm animals (reviewed in Charlier et al., 2014). Refugia for sensitive salmon lice, by leaving some of the fish untreated, have not been applied as a tool for delaying resistance development in salmon lice. Wild fish contributing to immigration of sensitive parasites may however have delayed the development of resistance. Immigration of sensitive parasites has been shown to reduce the rate of resistance development in insects (Georghiou and Taylor, 1977).

Drug resistance

Resistance has been defined by the World health organization (WHO) in 1957 as “the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species” (World Health Organization, 1957). Given that this definition described resistance at a population level and that resistance, according to this definition, could not be detected until after an unsuccessful treatment had been performed, several redefinitions have been made. The main goal has been to define resistance also at an individual level

in addition to having a definition that could be employed before the reduced treatment effect was observed. In an attempt to standardise definitions regarding resistance issues, Tabashnik et al. (2014) defined resistance as: “genetically based decrease in susceptibility to a pesticide”. In the current thesis the latter definition is applied. However many of the studies referred to are bioassay- or small-scale treatment studies on a single generation of parasites. The genetic component of the resistance observed in these studies is therefore assumed, but not proven.

Pesticide resistance in salmon lice is part of a worldwide problem with respect to resistant arthropods. These arthropods act as disease vectors or parasites on humans and animals or as crop pests. Control has become increasingly difficult due to emerging resistance. The strength of the resistance when practical/clinical (or field) resistance is seen may however differ between parasitic arthropods on fish and animals on one hand and crop pests or disease vectors on the other hand. This is due to the fact that the difference between the effective dose for sensitive parasites and the maximum applicable dose is often smaller in the former group due to the toxicity of the pesticide to the host of the parasite.

The distribution of resistance in salmon lice against the different chemical treatment agents is not fully known and is constantly changing. However several findings of resistance have been published. Pyrethroid resistance was first discovered by the use of biological assays (bioassays) on salmon lice from a farm reporting reduced treatment efficacy in Norway in 1998 (Sevatdal and Horsberg, 2000). Later resistance was confirmed through bioassay studies in other farms in Norway as well as in Ireland and Scotland (Sevatdal et al., 2005a). In the 2013 Norwegian resistance surveillance program for *L. salmonis*, simplified bioassays were performed, according to a protocol developed based on results from paper I. The presence of pyrethroid resistance was shown along the entire Norwegian coast with the exception of the far north (the far south was not included in the survey) (Grøntvedt et al., 2014). In Eastern Canada, pyrethroid treatment efficacy and bioassay results from 2009 and 2010 showed signs of resistance (Whyte et al., 2014). These treatment results may however also be due to suboptimally performed treatments (Beattie, 2009).

Organophosphate resistance was first found in Scotland in 1990 (Jones et al., 1992). From the same time period resistance towards this type of chemical was also seen in Ireland (Tully and McFadden, 2000) and in a second study from Scotland (Roth et al., 1996). All of these aforementioned studies were performed using various bioassay protocols. In the 2013 Norwegian national survey the azamethiphos resistance situation was seen to be very similar to the situation for pyrethroid resistance. With the exception of the far north, resistance was found distributed all along the Norwegian coast (Grøntvedt et al., 2014).

At the current point in time hydrogen peroxide resistance has been described in one small-scale treatment study from Scotland in 2000 (Treasurer et al., 2000c). Hydrogen peroxide resistance was found in Mid-Norway and in the south-western part of Norway in the 2014 Norwegian resistance surveillance program for *L. salmonis* (Grøntvedt et al., 2015). Resistance towards diflubenzuron or teflubenzuron has not yet been reported in salmon lice, although this type of resistance is present in other species (Ahmad et al., 2008).

Emamectin benzoate is commonly used in treatments of salmon lice throughout the salmonid producing world and resistance against this compound is widespread. In British Columbia however, EMB still seems to have good effect. This may possibly be due to the large number of wild, untreated

salmonid hosts from which sensitive parasites can immigrate into the farms (Saksida et al., 2010). In Eastern Canada, EMB treatment effect was reduced between the years 2004-2008 and this reduction was possibly due to resistance (Jones et al., 2012). The same tendency was observed for Scottish EMB treatments between the years 2002 and 2006 (Lees et al., 2008). The 2013 Norwegian survey showed a pattern of moderate resistance towards EMB, but the level of resistance may have been underestimated due to methodological errors (Grøntvedt et al., 2014). No fitness costs were found associated with EMB resistance in *L. salmonis* in a laboratory study (Espedal et al., 2013).

Resistant salmon lice are problematic for the fish farmers as they lose tools against the parasite. If all chemical tools are lost, the farmer may have to slaughter the fish ahead of schedule and leave locations fallowed for longer periods of time than planned. The primary choice when treatment efficacy is reduced is often increased frequency of the treatments as well as using higher concentrations of the chemical agent and/or increased exposure time. Indications of these actions can be seen in the increased use of chemicals compared to the production of salmonids that occurred in Norway from 2008 (Figure 3) (Helgesen et al., 2014). More frequently applied treatments are both expensive for the fish farmer as well as for the environment due to the increase in chemical discharge. Furthermore it accelerates the development of resistance as selection pressure for the same resistance trait is applied multiple times. Finally this measure implies a hazard for the fish as each treatment is accompanied by its potential dangers. Increased dosages and/or exposure time increase this risk, as the safety margins (the dose dangerous for the fish minus the treatment dose) are small for some of the treatment agents (reviewed in Roth et al., 1993).

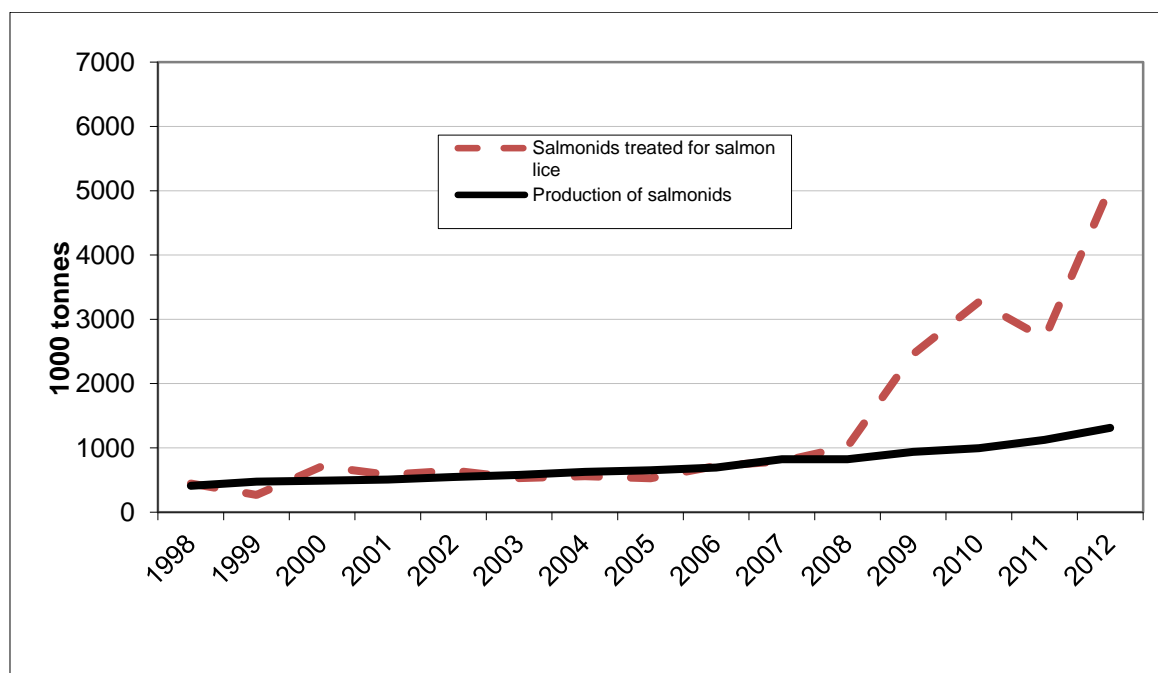


Figure 3. The solid black line shows the combined production of Atlantic salmon, rainbow trout and arctic char in Norway from 1998 to 2012. The stippled red line represents the biomass of these species treated against salmon lice with all available chemical treatments. See Helgesen et al. (2014) for assumptions made in the calculations. The data were collected from the Norwegian Institute of Public Health (<http://www.fhi.no/tema/legemidler/legemidler-i-fiskeoppdrett>) and Statistics Norway (<http://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett/aar-forelopige/2013-06-06?fane=tabell&sort=nummer&tabell=117233>).

Resistance testing and resistance mechanisms

Resistance testing is important in order to detect possible resistance prior to treatment as well as to investigate if a failed treatment is caused by resistant parasites or improperly performed treatment (Denholm et al., 2002). If resistance is determined, other treatment agents may be chosen in order to facilitate good treatment effect. A new and improved treatment with the same agent will only have a better effect if the salmon lice are sensitive towards the treatment agent. If the parasites have developed resistance, a second treatment with the same agent will most likely show even worse efficacy. This is due to the fact that the first treatment facilitated selection towards stronger resistance.

The changes in treatment efficacy might be used to analyse resistance development retrospectively. This has been done in Scotland and Canada with regards to EMB resistance (Jones et al., 2012; Lees et al., 2008). Other methods for resistance testing are however necessary to detect resistance in individual farms. This is due to the fact that treatment efficacy is dependent of a variety of factors besides resistance. Resistance testing is also essential in order to detect resistance at an early stage, when there are few resistant individuals or when the resistance level in each parasite is low. If resistance is detected at this stage in its development, early measures may be implemented in order to delay the development of resistance. These measures can subsequently prolong the efficacy period of the chemical treatment agent. This concept has been demonstrated in resistance in cotton pests (reviewed in Sawicki and Denholm, 1987).

Given that resistance testing in controlled small-scale treatments is both expensive and undesirable from an animal welfare perspective, bioassays are advantageous. Bioassays are toxicological tests where groups of live salmon lice, detached from the fish, are exposed to different concentrations of the test chemical. The bioassays applied on salmon lice are called binary quantal response experiments with one explanatory variable in the book “Bioassays with arthropods” by Robertson et al. (2007). The desired detectable binary quantal response is the number of parasites that are dead/immobilized or alive for each concentration of the chemical. The explanatory variable is the different concentrations of the test chemical. The results from the bioassay are modelled in a dose-response curve and the EC_{50} -value is determined (the concentration immobilising 50 percent of the parasites). This value is subsequently applied to describe the sensitivity level of the parasites.

Bioassays were first applied to test for resistance in *L. salmonis* towards organophosphates (Jones et al., 1992; Roth et al., 1996; Tully and McFadden, 2000). In these studies several different bioassay protocols were applied. An attempt to standardise bioassays was made for pyrethroid and EMB resistance testing of *L. salmonis*, but different protocols were developed for each of the two classes of chemicals (Sevatdal et al., 2005a; Sevatdal and Horsberg, 2003; Westcott et al., 2008). In order to simplify the EMB bioassay protocol, a fixed dose approach bioassay was developed (Whyte et al., 2013). A simplified bioassay protocol was also applied in the resistance surveys conducted in Norway in 2013 and 2014 (Grøntvedt et al., 2014; Grøntvedt et al., 2015).

At the onset of the current study no molecular assays existed to test for salmon lice resistance. This was due to the fact that the mechanisms behind resistance, towards any of the chemicals, had not been fully elucidated. Extensive research has however been invested in this area in recent years. The reason for this is that knowledge of resistance mechanisms could provide more accurate tools for resistance testing, also at an individual level, than the bioassays at use today.

Monoxygenase mediated pyrethroid detoxification has been shown in pyrethroid resistant salmon lice (Sevatdal et al., 2005b). A novel mutation in the voltage gated sodium channel gene has also been detected in salmon lice, from farms reporting of reduced pyrethroid treatment efficacy (Fallang et al., 2005). The parasites included in the study by Fallang et al. (2005) were however not individually selected for resistance and only one of the four domains of the gene were included in the study. With respect to other arthropods, several pyrethroid resistance mechanisms have been detected; a wide variety of mutations in voltage gated sodium channel genes as well as up-regulation of enzymes involved in pyrethroid metabolism or detoxification such as carboxylesterases, cytochrom P450s, glutathione-S-transferases and superoxide dismutases (Müller et al., 2007; Ranson et al., 2011; Soderlund and Knipple, 2003; Vontas et al., 2001; Xu et al., 2013). Reduced cuticular penetration has also been observed as a cause of pyrethroid resistance in arthropods (Ahmad et al., 2006). In *C. rogercresseyi*, an increased expression of genes, belonging to an antioxidant system, were observed in salmon lice, following exposure to deltamethrin. This observation indicates the involvement of this system in the detoxification of deltamethrin (Chavez-Mardones and Gallardo-Escárate, 2014). An increased P-glycoprotein (P-gp) expression was found in female *C. rogercresseyi* after pre-exposure to deltamethrin, suggesting that P-gp participate in pyrethroid metabolism (Valenzuela-Muñoz et al., 2014). However, none of the two aforementioned studies included control groups containing sensitive lice. Therefore an association between up-regulation of these genes and resistance could not be established.

With respect to organophosphate resistant salmon lice, biochemical evidence regarding the existence of a resistant type of acetylcholinesterase has been given (Fallang et al., 2004). This is in accordance with results from other organophosphate resistant arthropods where several mutations have been determined in acetylcholinesterase or carboxylesterase genes (Hotelier et al., 2010). Furthermore up-regulation of the expression of esterase genes, due to gene amplification, has also been shown to be a resistance mechanism in insects (reviewed in Bass and Field, 2011).

A polygenetic origin of EMB resistance was suggested in a Canadian microarray study by Sutherland et al. (2014b). This suggestion was based on the up-regulation of multiple genes in the resistant lice, including degradative enzymes such as collagenases. In a Scottish microarray study, decreased mRNA expression of the two ligand-gated ion channels GABA-gated chloride channel subunit and neuronal acetylcholine receptor subunit were found in EMB resistant *L. salmonis* in comparison to sensitive parasites (Carmichael et al., 2013). This finding could however not be demonstrated by Sutherland et al. (2014). Igboeli et al. (2012) showed that the ATP-binding cassette (ABC) transporter P-glycoprotein (P-gp) may be associated with EMB resistance in salmon lice. Their study showed that mortality in male parasites increased post-EMB exposure when pre-exposed to verapamil. Verapamil was assumed to be an inhibitor of ABC transporter in salmon lice. Furthermore increased mRNA expression of P-gp was shown and correlated to the EMB concentration, in surviving parasites from a bioassay. Another study by Igboeli et al. (2013) showed higher expression of P-gp in males than in females, in accordance with gender differences in susceptibility found in bioassays. The same study found increased expression of P-gp in males from a resistant strain in comparison to males from a sensitive strain. The difference in P-gp expression was not seen for the female parasites. Since only survivors from bioassays were applied, the studied individuals from the sensitive strains might however also have been resistant. Verapamil was also found to reduce the resistance level of EMB resistant salmon lice in a study carried out by Heumann et al. (2014). In another study by Heumann et al. (2012) they did not find any differences in P-gp expression levels between resistant and

sensitive parasites when unexposed to EMB. Following aqueous EMB exposure they reported an increase in P-gp expression in resistant parasites. The same increase was however not seen in the resistant parasites exposed to the chemical by EMB treatment of the fish.

Despite the fact that the mechanisms for EMB resistance in salmon lice are not fully known, the spread of this resistance has been elucidated through a single nucleotide polymorphism (SNP)-array study. This study showed the same conserved haplotype, associated with EMB resistance, throughout the Atlantic Ocean. The same study also indicated two regions in the salmon lice genome where the resistance mechanism may possibly be found (Besnier et al., 2014). In other species, mutations in the glutamate gated chloride channel, increased metabolism by mixed function oxidases, carboxylesterase or glutathione S-transferase, and increased expression of the ATP binding cassette (ABC) transporters P-glycoproteins and multi-drug resistance proteins, have been observed to cause avermectin resistance (reviewed in Wolstenholme and Kaplan, 2012).

No resistance mechanisms to combat hydrogen peroxide or the benzoylureas have been reported in salmon lice. In *Tetranychus urticae* mutation in chitin synthase has been associated with benzoylureas resistance (reviewed in Van Leeuwen et al., 2010). An increase in catalase and glutathione peroxidase has been determined in hydrogen peroxide resistant mammalian cells (Baud et al., 2004; Spitz et al., 1992). Increased catalase levels have also been found in fungus and bacteria in association with H₂O₂ resistance (Amin and Olson, 1968; Mutoh et al., 1999).

AIMS OF THE STUDY

Overall aim:

To refine existing methods and develop new techniques for monitoring of drug resistance in the salmon louse *L. salmonis*.

Sub-goals:

- 1) To develop a simpler bioassay protocol for resistance testing of salmon lice towards the commonly used chemotherapeutants; deltamethrin, cypermethrin, azamethiphos and emamectin benzoate.
- 2) To develop bioassay protocols suitable for resistance testing of salmon lice towards hydrogen peroxide and to apply these to selected salmon louse strains.
- 3) To elucidate the molecular mechanism behind azamethiphos resistance in salmon lice and to develop a rapid high-throughput resistance assay to detect this mechanism.
- 4) To elucidate the molecular mechanism for pyrethroid resistance in salmon lice and to develop a rapid high-throughput resistance assay to detect this mechanism.

METHODS

Collection of salmon lice (all papers)

Salmon lice from the field were collected at fish farms or processing plants in Norway. The farmers had agreed to the lice being used for research purposes. The salmon louse strains were collected based on their assumed sensitivity towards the different chemical treatment agents. Information of their sensitivity was collected from treatment efficacy data and field bioassays. The lice were collected as pre-adults, adults or egg-strings. At the fish farms, fish were netted from the cages and anaesthetised in a bath before salmon lice were manually removed. The fish were allowed to recover in clean sea water before they were put back into the cages. At the processing plants salmon lice were removed after the fish had been anaesthetised, but prior to bleeding.

The salmon lice were transported in cooled sea water and used for bioassays directly or shipped to Oslo via postal delivery. In the case of shipment, the salmon lice were put in sea water filled bottles surrounded by cooling elements.

Salmon lice from the laboratory were picked off anaesthetised fish when the lice had reached the appropriate instar for the study or after a certain post-treatment time interval.

Cultivation of salmon lice (all papers)

The different salmon louse strains were kept in continuous culture at the The Norwegian Institute for Water Research's marine research station in Drøbak (NIVA) or at the University of Bergen (UiB). The fish, used as hosts for the parasites at NIVA, were Atlantic salmon from the commercial supplier Sørsmolt in Kragerø or rainbow trout from the University of Life Sciences at Ås. Atlantic salmon, from the Institute of Marine Research's breeding station in Matre, were applied as hosts for the parasites at UiB.

Fish were infested with salmon lice copepodites in bath trials. Here the fish were kept in a reduced water volume without water circulation combined with a predetermined number of copepodites, for 15 - 60 minutes. Additional aeration was supplied and the fish were kept under constant surveillance during the infestation. The fish were returned to their tanks with normal water circulation after the infestation. When infested with adult parasites, the fish were anaesthetised and the parasites manually placed on them.

The number of salmon lice on the fish and their present instar was regularly investigated by examination of a selection of fish. Parasites were, if necessary, removed from the fish as pre-adults to avoid over-infestation of the fish with adult lice. Egg-strings from adult females were removed from the lice while they were still attached to the fish.

Egg strings were put into a salmon louse hatchery (figure 4) where they were maintained in plastic tubes with constant water circulation. Following the development of the larvae into copepodites, the copepodites were used for infestation trials. Further details on the hatchery are given in Hamre et al. (2009).

The fish were anaesthetised prior to all treatment procedures, using benzocaine (supplied by Norsk Medisinaldepot) dissolved in ethanol, at a concentration of 80 mg L⁻¹ sea water or Finquel vet (Tricain

mesilat, Western Chemical Inc., USA) dissolved in fresh water, at a final concentration of 125 mg L⁻¹ sea water. Fish were euthanized in an anaesthesia bath containing an overdose of the same substances.



Figure 4.
Hatchery

Photo: Stian Mørch Aaen

Small-scale treatments (paper I, III and IV)

Small-scale treatments of fish, infested with salmon lice, were performed to assess treatment efficacy of a given chemical. Other objectives included the selection of resistant parasites as well as genotyping affected and unaffected parasites from a treatment. Bath treatments were performed on fish infested with pre-adult or adult lice, while the in-feed treatment was performed on fish infested with salmon lice in the chalimus stage. Treatments were performed on fish in their original tanks. For the treatment efficacy trials, fish held in the same type of tanks and infested with salmon lice in the same infestation trial were subjected to a sham treatment and acted as the control group. The fish were allocated randomly in treatment and control groups.

Bath treatments with deltamethrin and azamethiphos were performed according to the manufacturer's recommendations. For deltamethrin treatments ALPHA MAX (deltamethrin 10 mg ml⁻¹, Pharmaq AS, Norway) was applied at a concentration of 2 mg deltamethrin/1000 L sea water. For azamethiphos treatments, Salmosan (50 % w/w azamethiphos, Fish Vet Group, UK) was used at a concentration of 0.1 g azamethiphos per 1000 L sea water. Bath treatments were performed by stopping the water circulation but allowing aeration of the fish tanks before adding the appropriate amount of treatment agent. The water was drained from the tank after 30 minutes exposure until the dorsal fin of the fish was above water level. The tanks were subsequently refilled and normal water circulation was established. The treatment effect was evaluated 5-8 days later by counting lice on all treated and control fish.

For one of the azamethiphos treatments in paper III, the parasites which fell off the fish were immediately picked out of the tank for the first 2.5 hours following the initiation of the exposure. Detached parasites were also picked out of the tank 24 hours later. Eight days after exposure all remaining parasites were removed from the fish.

In-feed treatment with EMB was performed by feeding two tanks of fish 0.5 % of their body weight per day for a week. One tank was fed pellets medicated with Slice (EMB 10 mg/kg, MSD, USA) giving each fish 50 µg EMB per kg bodyweight per day. The control fish were fed their regular feed. For the following two weeks, both tanks were fed their regular, non-medicated feed ad libitum. The number of parasites on each fish was recorded two weeks after the end of the treatment period.

Bioassays (paper I – III)

Groups of pre-adult and adult male salmon lice, removed from the fish, were exposed to different concentrations of the treatment agents in order to determine the sensitivity level of each strain to each individual chemical. One group was kept as a control group and was not exposed to the treatment. The other groups were exposed to varying concentrations of the chemical dependent on which treatment agent was applied, the exposure time and the strain of salmon lice tested. Between six and twelve different concentrations of the test chemical were applied for each bioassay. This also included a control group exposed to sea water. The aim was to expose the parasites to a single concentration which would kill all parasites, while the remained of the concentrations should serve to give mortality rates between 0 and 99 %. At the end of the experiment, the salmon lice were classified as either alive or immobilized/dead. The number of alive and immobilized/dead parasites for each concentration was then modelled and the EC_{50} -value was calculated based on these modelled results.

Bioassays were performed on deltamethrin using ALPHA MAX, hydrogen peroxide using Interlox Paramove 50 (H_2O_2 50 % w/w, Solvay Chemicals, Belgium) or Eka HP T49 S (H_2O_2 49.7 % w/w, Azko Nobel, Netherlands), azamethiphos using analytical standard (Sigma-Aldrich, USA), or EMB using analytical standard (Schering-Plough, USA). The two latter substances were dissolved in ethanol to prepare stock solutions. Working solutions of the chemicals diluted in sea water were prepared immediately prior to use.

The parasites were placed in either polystyrene boxes (2.5 x 2 x 8 cm (0.04 L)) for the traditional bioassays or in one litre glass flasks containing fresh sea water for the simplified bioassays. The boxes were fenestrated at each end. The parasites were allocated evenly and randomly between the containers; approximately 10 parasites in each box or 30 parasites in each flask. The boxes were submerged into one litre sea water baths containing a given concentration of the bioassay chemical, while the correct amount of the chemical was added to the flasks to achieve the correct concentrations. After 30 minutes (deltamethrin and hydrogen peroxide), 60 minutes (azamethiphos) or 24 hours (EMB) the boxes were taken from the chemical bath and rinsed in fresh sea water. The results were evaluated immediately after exposure for both hydrogen peroxide and EMB or after 24 hours at 12 °C in fresh, aerated sea water for pyrethroids and azamethiphos. The hydrogen peroxide bioassays were re-evaluated after 24 hours in fresh sea water. In the glass flasks the exposure time was 24 hours for all treatment agents and the results were evaluated at the end of the exposure period.

Parasites were regarded as alive if they were able to stick to the surface of the container or swim in a straight line. This was tested for each individual in the traditional bioassay. In the simplified bioassays all parasites still attached to the bottle wall when the water was poured out was regarded as alive. The parasites poured out were regarded as immobilized in paper I while they were evaluated individually in paper II by pouring the content of the bottle into a beaker.

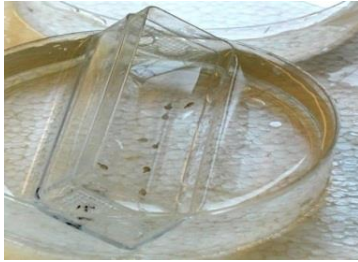


Figure 5.
Bioassay box

Photos: Tor Einar Horsberg



Figure 6.
Glass flask for bioassays

Molecular methods (paper III and IV)

Molecular studies were performed on the voltage gated sodium permeable channel (Na_v) genes $\text{Na}_v1.1$, $\text{Na}_v1.2$ and $\text{Na}_v1.3$ and the acetylcholinesterase (AChE) genes *ace1a* and *ace1b* from the salmon louse.

In an early assembly of the salmon louse genome (<http://sealouse.imr.no/>), several putative Na_v contigs were identified and these were used to design primers for rapid amplification of complementary DNA (cDNA) ends (RACE). This was required in order to obtain the full-length cDNA sequence of $\text{Na}_v1.1$. A protein blast in LiceBase (<https://licebase.org/>) was performed using the putative $\text{Na}_v1.1$ amino acid sequence as a template in order to identify the genes $\text{Na}_v1.2$ and $\text{Na}_v1.3$ in the salmon louse genome. The entire cDNA sequence of *ace1a* and *ace1b* was obtained through RACE PCR in a study by Kaur et al. (2015).

A quantitative polymerase chain reaction (qPCR) study was performed on the $\text{Na}_v1.1$, $\text{Na}_v1.2$ and $\text{Na}_v1.3$ genes. The elongation factor gene was used as the reference gene. The qPCR analysis was carried out in 96 well plates on BioRad CFX96 real-time system. Each sample was run in triplicates for each gene and the mean Ct values for each gene and sample was calculated. The calculated means were then used for normalization of the expression according to the formula $\Delta\text{Ct} = \text{Ct}_{\text{reference}} - \text{Ct}_{\text{target}}$.

PCRs were performed with cDNA as template to create amplicons for the whole gene sequence of *ace1a* and *ace1b* or partial gene sequences of $\text{Na}_v1.1$. Amplicons from PCR reactions were subjected to in-house direct sequencing or external sequencing by Eurofins MWG Operon, Germany. Sequence analysis were performed in VectorNTI (Informax Inc, USA) or Sequencher 4.10.1 (Gene Codes Corp., USA)

A high resolution melt (HRM) analyses tool was developed in house and a TaqMan assay was developed by PatoGen Analyse AS, Ålesund, for the rapid screening of the mutation *Phe362Tyr* in *ace1a*.

Gene studies (paper III and IV)

The complete cDNA sequence for $\text{Na}_v1.1$ was blasted against the *L. salmonis* genome in order to find the organization of the gene in *L. salmonis* using the Spidey mRNA to genomic alignment program (<http://www.ncbi.nlm.nih.gov/spidey/>). The genomic organization of $\text{Na}_v1.2$ and $\text{Na}_v1.3$ was found directly in the salmon louse genome.

The Na_v and AChE genes of the salmon louse were aligned with Na_v or AChE genes from other species collected in GenBank (GB) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), using the alignment tool on the website of the European molecular biology laboratory/European bioinformatics institute (EMBL-EBI) (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Na_v and AChE genes were aligned separately. A phylogenetic tree was constructed for the Na_v genes using MEGA6 (Tamura et al., 2013).

The three-dimensional structure of the enzyme *ace1a* was modelled using SWISS MODEL in the automated mode ((Arnold et al., 2006); <http://swissmodel.expasy.org/>). The models were generated using native AChE from *Drosophila melanogaster* as templates. The template was generated on basis of the crystalline structure of the *D. melanogaster* AChE proteins (Harel et al., 2000) and the best fit with this template was found.

Enzymatic assays (paper III)

Biochemical AChE assays were performed on pre-adult female salmon lice. AChE was inhibited *in vitro* in lice homogenate using propoxur, an AChE inhibitor classified as a carbamate, or *in vivo* subjecting live parasites to azamethiphos. The enzymatic activity was analysed using the principle of Ellman et al. (1961) on 96 wells microtitre plates. A slightly modified version of a protocol developed by the World Health Organization (WHO) to detect pesticide resistance mechanisms in insects was used (World Health Organization, 1998a).

Statistical analysis (all papers)

All statistical analysis was performed using the statistical software JMP 10 (SAS Institute Inc., USA) if not specified otherwise. The bioassay results were modelled using four-parametric non-linear regression or probit analysis and EC₅₀-values with confidence intervals were calculated. The mean treatment efficacies in the small-scale treatments were calculated using bootstrapping with 2500 simulations calculating the difference (in %) in the number of parasites between the treated groups and their respective control groups. The 95 % confidence intervals (CI) for the efficacy were constructed using the number of fish per treated group as N. A t-test was applied to test for differences in treatment efficacies between the strains for each treatment chemical using the online tool <http://www.graphpad.com>.

From the small-scale treatment with azamethiphos in paper III, on the salmon louse strain Ls F, an analysis of survival versus time for the three genotypes of *ace1a* was conducted with a Kaplan-Meier survival analysis including a Wilcoxon test.

Analyses of Δ Ct-values from the qPCR study on the Na_v genes were made by repeated measures analysis of variance (ANOVA). The factors were gene, strain and salmon lice number. The two-way interaction between strain and gene was included in the model. The post hoc test of the effect of gene was conducted with a Tukey HSD test. The post hoc test of the effect of strain for each gene was conducted using a Dunnet test with the sensitive strain as the control.

The results from the enzymatic AChE inhibition studies were statistically compared with ANOVA after root transformation (*in vitro* study) or the non-parametric multiple comparison Steel-Dwass method (*in vivo* study).

MAIN RESULTS

Paper I: Simplified bioassays

Given that the existing 6-dose bioassay protocols for resistance testing in *L. salmonis* were time consuming to perform, required skilled personnel to carry out and evaluate and had not been validated regarding their association to treatment results, a new bioassay protocol was developed. The goal was to develop one bioassay protocol suitable for all treatment agents. The protocol should be easier and less time consuming to perform as well as requiring fewer parasites than the original protocols. The former bioassay protocols were created to detect salmon lice resistance in laboratory reared parasites, while the present protocol was intended to detect resistance in field collected parasites and possibly be further developed in order to allow for the prediction of treatment efficacy.

A sensitive strain and a strain showing reduced sensitivity were identified for each chemical after performing traditional bioassays and small-scale treatments. The EC₅₀-results from the traditional bioassays showed a 2.4–19 times difference in deltamethrin sensitivity between the strains, a 10.4–13.0 times difference in azamethiphos sensitivity and a 4.7–5.9 times difference in EMB sensitivity. The small-scale treatments showed a difference in deltamethrin treatment effect between the sensitive and the resistant strains of 24.1–25.9 %, in azamethiphos a difference in treatment effect of 50.2 % and a difference in EMB treatment effect of 11.4 %. All differences found in the small-scale treatments were statistically significant ($p < 0.05$).

The newly developed bioassay protocol differentiated between the sensitive and the resistant strains for each chemical in accordance with the results from the traditional bioassays and the small-scale treatments. There was a 19–24.5 times difference between the strains in the EC₅₀-values obtained from the 24-hour deltamethrin bioassays, a 31 times difference in the azamethiphos bioassays and a 7.8 times difference in the EMB bioassays. The bioassay results from both traditional bioassays and 24-hour bioassays are presented in Table 1. The greatest difference, in the 24-hour bioassay, between the dose-response curves for the lower 80 % prediction interval for the sensitive strain and the upper 80 % prediction interval for the strain showing reduced sensitivity, was identified for each delousing agent. The corresponding concentration of the chemicals, at which the greatest difference in mortality was found, was subsequently established. This concentration was termed the optimal separating dose. In the dose-response curves for the 24-hour deltamethrin bioassays, the greatest difference in mortality was 77.6 %. This difference was calculated at a concentration of 0.04 $\mu\text{g L}^{-1}$. With respect to azamethiphos the greatest difference was 44.9 %, which corresponds to a calculated concentration of 0.3 $\mu\text{g L}^{-1}$. With regards to EMB, the largest difference was 63.5 %, which was calculated for a concentration of 47 $\mu\text{g L}^{-1}$.

Several attempts to validate this protocol with regards to field treatment efficacy have been made (not published). As part of this validation process a bioassay with 24 hours exposure to deltamethrin was run in the County of Hordaland and resulted in a control group mortality of 20 %, a mortality of 8.3 % in a group exposed to 0.05 $\mu\text{g L}^{-1}$ and a 15.8 % mortality in a group exposed to 0.2 $\mu\text{g L}^{-1}$. The treatment result from a deltamethrin treatment performed at the farm two weeks later showed a treatment efficacy of 71 % in pre-adults and adult males while 91 % mortality was observed in adult females.

Strain and chemical	Traditional bioassays			24-hour bioassays		
	EC50	90 % CI low	90 % CI high	EC50	90 % CI low	90 % CI high
Deltamethrin						
Ls A (sensitive)	0.20	0.15	0.24	0.02	0.02	0.02
Ls A	0.72	0.50	1.10			
Ls B (resistant)	2.79	1.39	150	0.38	0.24	0.54
Ls B	3.82	3.41	4.48			
Ls R (resistant)	1.73	1.41	2.18	0.49	0.41	0.57
Azamethiphos						
Ls A (sensitive)	1.01	*	*	0.12	0.11	0.14
Ls H (resistant)	10.5	7.31	61.1	3.72	2.14	11.5
Ls H	13.1	5.86	132			
Emamectin benzoate						
Ls A (sensitive)	51.4	*	*	21.5	18.2	23.7
Ls B (resistant)	243	127	409	167	138	199
Ls B	302	*	*			

Table 1. Results from traditional bioassays and 24-hour bioassays conducted on four different strains of salmon lice. The concentrations immobilizing 50 % of the parasites (EC50) given in $\mu\text{g L}^{-1}$, with adhering two-sided 90 % confidence intervals (CI), are presented. An asterisk in the table indicates that the CI could not be calculated.

Paper II: Hydrogen peroxide resistance

Paper I did not include hydrogen peroxide as reports of reduced treatment efficacy of this chemical had not been received at the time of the study. When reduced treatment efficacy from H_2O_2 treatments was reported, a bioassay protocol was of the utmost importance in order to determine if the reduced treatment efficacy was due to erroneously performed treatments or due to hydrogen peroxide resistant parasites.

The results show that bioassays permit differentiation between strains of salmon lice with regards to H_2O_2 -sensitivity. Furthermore this coincides with treatment efficacies. Following a 30 minutes exposure, the strain with a history of reduced treatment efficacy (Ls V-F0) showed an EC_{50} -value of 2127 mg L^{-1} . The strains from the surrounding area that had been treated successfully with H_2O_2 showed EC_{50} -values of between 541 and 693 mg L^{-1} . The progeny of the least sensitive salmon lice (Ls V-F1) also showed reduced H_2O_2 sensitivity in a bioassay, with an EC_{50} -value of 1767 mg L^{-1} . The strain from the far north of Norway (Ls A) which had never been treated with H_2O_2 , showed an EC_{50} -value of 216 mg L^{-1} , giving that up to ten times difference in sensitivity between two strains was recorded. The modelled results from Ls V-F1 and Ls A are displayed in figure 7.

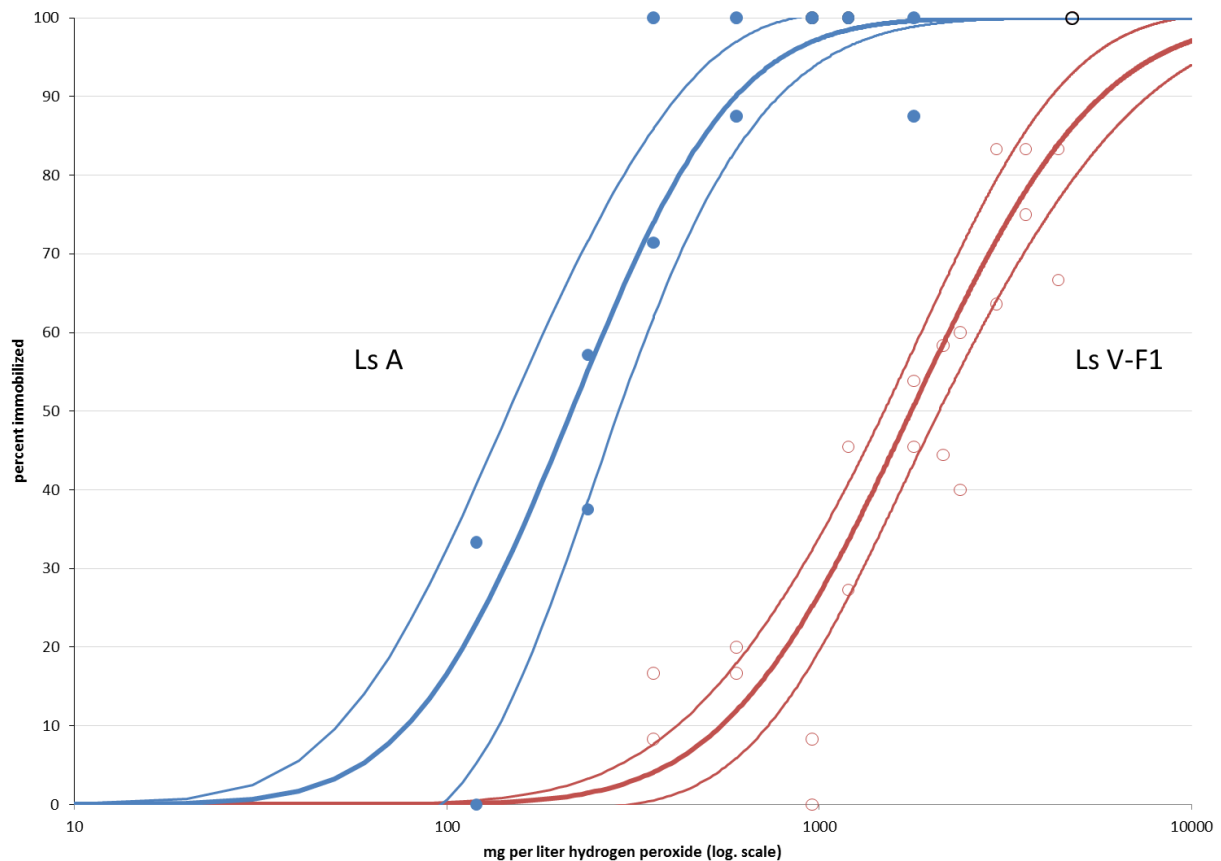


Figure 7. Bioassay results from Ls V-F1 and Ls A following 30 minutes exposure to H₂O₂. The dots represent the individual results while the lines with 90 % confidence intervals were modelled using probit modelling.

The 24 hours exposure to H₂O₂ also allowed differentiation between the salmon lice strains with regards to their sensitivity. Ls V-F1 showed EC₅₀-values of 138 mg L⁻¹ while Ls A showed EC₅₀-values of 45.9 and 64.7 mg L⁻¹. The differences between these two strains were also observed when the parasites, which had been exposed to H₂O₂ for 30 minutes, were re-evaluated after 24 hours in fresh sea water. These results could however not be modelled due to illogical dose-response curves. In the re-evaluation the percentage of parasites regarded as immobilized or dead decreased from 48 % to 21 % in the resistant strain, while the sensitive strain showed a decrease from 68 % to 51 %.

In an unpublished study, where we looked at gender differences in susceptibility towards H₂O₂, EC₅₀-values of 1192 mg L⁻¹ were seen for adult males and 359 mg L⁻¹ for pre-adult II females. The bioassay was performed as a 30-minute H₂O₂ bioassay on a laboratory reared F1-generation. The salmon lice originated from a farm in the south-western part of Norway which had reported of reduced treatment efficacy after H₂O₂ treatments.

In another of our unpublished studies the expression level of the enzyme catalase was investigated in a qPCR study using primers designed for the catalase gene in *L. salmonis*. Parasites from Ls V and Ls A from paper II were included. A significant overexpression of this enzyme was found in the resistant strain when compared to the fully sensitive strain. The same study also investigated the enzymatic activity of catalase in the two respective salmon louse strains. The results showed higher catalase activity in Ls V in comparison to Ls A.

Paper III: Acetylcholinesterase and organophosphate resistance

As is the case with all bioassays, the bioassay protocol developed in paper I to detect organophosphate resistance was attached with confounding factors. In order to develop a high-throughput method for organophosphate resistance testing, the resistance mechanism first needed to be elucidated. Given that AChE is a known organophosphate target and that AChE mutations are known resistance mechanisms in other arthropods, the study focused on these genes.

First the resistance status of different salmon louse strains were determined using bioassays and small-scale treatments. Thereafter the *L. salmonis* AChE genes (*ace1a* and *ace1b*) in two salmon louse strains, one sensitive (n=5) and one resistant (n=5) towards azamethiphos, were screened for mutations. This resulted in the identification of a missense mutation (*Phe362Tyr*) in *ace1a* in all lice from the resistant strain and a nonsynonymous single nucleotide polymorphism (*Ile433Thr*) in *ace1b*. The results from a further screening for the mutation in 100 parasites from two sensitive strains and 144 parasites from three resistant strains are displayed in Table 2. In one of the resistant strains surviving parasites following a small-scale azamethiphos treatment were also included. The results show a dispersion of the mutation coinciding with the organophosphate resistance of the respective salmon louse strain. The *Phe362Tyr* mutation was also found in parasites collected in 1998. The mutation was found in all survivors from an azamethiphos bioassay (n=4), while none of the immobilised salmon lice from the same assay had the mutation (n=5).

Strain	N	Sensitivity	Wild type Phe362/Phe362 frequency (SS)	Heterozygote Phe362/362Tyr frequency (RS)	Homozygote 362Tyr/362Tyr frequency (RR)
Ls A	50	Sensitive	100 %	0 %	0 %
Ls G	50	Sensitive	96 %	4 %	0 %
Ls B	50	Reduced sensitivity	72 %	26 %	2 %
Ls H	50	Resistant	44 %	36 %	20 %
Ls H-s	24	Resistant	0 %	92 %	8 %
Ls V	20	Resistant	5 %	35 %	60 %

Table 2. Frequency of the mutation *Phe362Tyr* in *ace1a* in different strains of salmon lice. Ls H-s were parasites from Ls H surviving an azamethiphos small-scale treatment.

In the alignment of the *L. salmonis* AChE genes with other species' AChE genes, the mutation was found to be in a highly conserved region of the gene. 3D modelling revealed that phenylalanine substitution with tyrosine at position 362 narrowed the entrance to the enzyme's catalytic triad and changed the polarity in the gorge. In the small-scale azamethiphos treatment trial for genotyping, of both detached and surviving parasites, all wild type parasites (SS) died from the treatment (n=10), 44 % of the heterozygote (RS) died (n=25) while none of the homozygote for the mutation (RR) died (n=16). In the enzymatic assay where the residual activity of AChE were measured, following in vitro inhibition with propoxur, the sensitive lice were significantly more inhibited than the resistant

lice ($p < 0.0001$). The *in vivo* inhibition with azamethiphos was also significantly greater in the sensitive parasites ($p = 0.019$).

Based on these results we could strongly conclude that *Phe362Tyr* was responsible for the resistance in *L. salmonis* towards azamethiphos. Two rapid diagnostic assays were developed for the high-throughput screening of the *Phe362Tyr* mutation: A high resolution melt analysis (HRM) was developed in the present study, while Patogen Analyse AS in Ålesund used the results to develop a TaqMan assay. Both assays successfully discriminated between the three different genotypes and thereby rationalized genotyping in salmon lice compared to gene sequencing.

Paper IV: Sodium channels and pyrethroid resistance

As target sites for chemical treatment agents are highly specific, the high-throughput resistance assay, developed in paper III for organophosphate resistance, could not be used for pyrethroid resistance testing. A new study was therefore initiated in order to clarify the resistance mechanism for pyrethroid resistance in salmon lice. Given that voltage gated sodium channels (Na_v) are known target sites for pyrethroids in various arthropods, that several mutations in Na_v genes had been shown to cause pyrethroid resistance in other species and that a mutation in a Na_v gene had previously been described in *L. salmonis*, the study targeted these genes.

A complete cDNA sequence of the gene $Na_v1.1$ was obtained using RACE PCR. Two other putative Na_v genes were found; $Na_v1.2$ and $Na_v1.3$, by a homology search in the salmon louse genome using $Na_v1.1$ as the query. Characteristic features known from other Na_v genes were found in these three genes and they were therefore assumed to be the Na_v genes of *L. salmonis*. Although $Na_v1.2$ and $Na_v1.3$ were not highly similar to $Na_v1.1$ (51-57 % identity between $Na_v1.1$ and the two other genes in both nucleotide sequence and deduced amino acid sequence), they all clustered in the phylogenetic tree. The insect Na_v genes included in the alignment for the phylogenetic tree clustered in a different clade even though their amino acid sequences showed up to 66 % identity to $Na_v1.1$.

A qPCR study on the three Na_v genes was performed on 25 salmon lice from five strains (four resistant and one sensitive towards deltamethrin). Each strain was represented by five parasites. This study identified $Na_v1.1$ as the most expressed gene while $Na_v1.2$ was identified as the least expressed gene. There were significant differences between the expressions of all three genes ($p < 0.05$). The expression of each gene from each of the resistant strains was compared to the expression of the respective gene in the sensitive strain. The only significant difference determined was an overexpression of $Na_v1.2$ in two of the resistant strains. However this was not seen in the two others.

Since $Na_v1.1$ was the predominantly expressed Na_v gene, it was selected for the screening study. Homologous sequences to mutation hot-spots known from other arthropod species were identified in $Na_v1.1$. Screening for mutations in these hot-spots, using PCR and direct sequencing, were performed in 46 salmon lice from four different areas of Norway, all survivors of deltamethrin laboratory treatments. No mutations were identified when compared to the sequences collected from a fully sensitive strain of salmon lice. However, three synonymous single nucleotide polymorphisms (SNP) were identified in the sequences included in the study. These SNPs displayed a great heterogeneity among the studied salmon lice. The sequencing results are summarized in table 3.

Salmon louse		Mutations			Single nucleotide polymorphisms (SNP)		
					cc/ct/tt		
Strain	N	Domain 1	Domain 2	Domain 3	1712	1730	4622
Ls A	10	0	0	0	0/0/10	10/0/0	0/0/10
Ls D	15	0	0	0	6/5/4	4/7/4	6/4/5
Ls V	15	0	0	0	5/6/4	4/6/5	5/6/4
Ls Fr	5	0	0	0	2/2/1	2/2/1	2/3/0
Ls Fu	11	0	0	0	2/7/2	3/6/2	1/7/3
Total	56						

Table 3. Results obtained from the gene $Na_v1.1$ through PCR and direct sequencing of the amplicons. The number of samples from each strain and the number of mutations in each domain are displayed. The distribution of the three single nucleotide polymorphisms (SNP) is also shown. The salmon lice form Ls A was sensitive to pyrethroids, while the other salmon lice were individually selected for resistance. The SNPs were numbered (1712, 1730 and 4622) based on the nucleotide number in the $Nav1.1$ gene in salmon lice. cc: homozygote for cytosine, ct: heterozygote, tt: homozygote for thymine.

In a follow-up study (not published) additional primers were designed to cover more of the $Na_v1.1$ gene. Combined with the regions screened in paper IV, 95 % of the gene sequence was covered in 5 resistant parasites. No mutations were identified.

DISCUSSION

Aim 1: To develop a simpler bioassay protocol for resistance testing of salmon lice towards the commonly used chemotherapeutants; deltamethrin, cypermethrin, azamethiphos and emamectin benzoate

Resistance testing of salmon lice has been performed in several ways, including evaluation of treatment results, small-scale treatments and numerous different bioassay protocols. At the onset of the project, the salmon farming industry expressed an interest in a modified bioassay protocol. This was due to the fact that they needed a simple and reliable tool in order to determine field resistance. The aim of paper I was to develop a simple bioassay protocol which incorporated all the four different treatment chemicals; deltamethrin, cypermethrin, azamethiphos and emamectin benzoate. This was attempted achieved by the unification of the protocols for all test substances as far as it was possible. Furthermore, the goal was to reduce the number of concentrations of each chemical applied in a bioassay, thereby reducing the number of parasites needed. Additional means of achieve the overall aim included the standardizing of equipment used for the assays as well as simplification of the result evaluation in order for it to require less expertise.

In discriminative dose bioassays, parasites are exposed to a single concentration of a test chemical for a given period of time. Both the dose and time have been calibrated with the aim of killing all sensitive parasites. Surviving parasites are regarded as resistant. However the assay does not provide information about the level of the resistance (reviued in Corbel and N'Guessan, 2013). This type of assay is currently used for monitoring resistance in mosquitoes and is performed according to the protocols of either the World Health Organization (WHO) or the Centers for Disease Control and Prevention (CDC) (Centers for Disease Control and Prevention, 2015; World Health Organization,

1998b). Furthermore discriminative dose bioassays are also applied to test for resistance in crop pests and animal parasites (Kwon et al., 2010; Rust et al., 2005).

To develop discriminative dose bioassays, Robertson et al. (2007) recommended determining the concentration which kills 90-95 % of the parasites in the sensitive population(s) in multi-dose bioassays. This concentration should then be multiplied two or three times in order to determine a discriminating dose or array of doses. The WHO standard for determining the discriminative dose is to double the concentration killing 99 % of the sensitive parasites (LC_{99}) (World Health Organization, 1998b). In the initial work to find the discriminative doses for the WHO assays, bioassays on thousands of parasites from several sensitive strains were performed. In paper I only one sensitive strain was applied, due to limited availability of sensitive parasites and limited resources, to find the discriminative doses. The full natural variation in tolerance among sensitive parasites may therefore not have been identified and the doses might have been set too low.

The assays developed in paper I, may be regarded as modified discriminative dose bioassays. With respect to each treatment chemical in paper I, the optimal separating dose, between the sensitive and the resistant salmon louse strains, was identified. The experiments are described in detail in paper I. These optimal separating doses were considered appropriate for further use in field experiments, to study if the discriminative doses could predict field resistance.

However a comparison of these doses with treatment efficacy data from both laboratory- and field treatments, revealed that the relationship between these two factors was suboptimal. The unpublished results from Hordaland, described in the results section of this thesis, were similar to the results from the deltamethrin resistant strains, Ls B and Ls R, in paper I. These results showed that despite the fact that less than 20 % of the parasites died following exposure to discriminating concentrations of deltamethrin, more than 70 % of the parasites died in a deltamethrin treatment performed after the bioassay. It may therefore be surmised that salmon lice with a certain degree of reduced sensitivity can still be killed by a treatment. As a result the bioassay concentrations applied in Hordaland could not be used to predict treatment efficacy. The concentrations could however be used to distinguish between fully sensitive strains and strains with reduced sensitivity. From the dose-response curves of the resistant strains it was possible to assume that exposure of the parasites to the optimal separating doses in azamethiphos and EMB bioassays would also show lower mortalities than the mortalities observed in the small-scale treatments. As a result these bioassay concentrations would most likely overestimate the level of field resistance. When the bioassay protocol for the Norwegian surveillance program (Grøntvedt et al., 2014) was developed, an additional concentration, which was higher than the concentration used to discriminate between fully sensitive parasites and parasites with reduced sensitivity, was added to the test protocol. Furthermore the lowest concentration applied was increased compared to the doses described in paper I. This was in accordance with the suggestion of Robertson et al. (2007).

A validation process between bioassay and treatment results is a challenging task given that several aspects of bioassays, treatments and treatment evaluations may be attached with confounding factors. With regards to the bioassays, sampling procedures for the salmon lice as well as handling of the lice during the bioassays are important in order to avoid salmon lice being killed for reasons other than the chemicals applied in the assays. Natural variation in sensitivity amongst the parasites creates uncertainty as to how well each selection of lice represents the sensitivity of the whole

population (Robertson et al., 1995). The CDC recommended exposing 100 mosquitos to the discriminative dose (Centers for Disease Control and Prevention, 2015). Testing this amount of salmon lice would increase the number applied in the bioassay compared to the traditional six-dose bioassay. However, fewer parasites render the question of representativeness more pertinent.

In the case of differences in susceptibility between the gender and instar of the parasites, these two factors become important for the bioassay results. The pyrethroid sensitivity has been shown to be similar in both pre-adult I and II and adult males. Adult females have however shown lower sensitivity (Sevatdal, 2005). With regards to EMB, pre-adult males have shown lower sensitivity than females (Westcott et al., 2008). In a treatment trial, sensitivity towards azamethiphos was found to differ according to parasite size. The smallest parasites were the most sensitive (Roth et al., 1996). Further studies are however required in order to fully understand the differences in sensitivity between the instars and the two genders of salmon lice. The use of only one specific instar and a certain gender in the bioassays will, however, make it difficult to use these bioassays in the field. An alternative approach is to introduce correction factors to account for these differences. This is however only possible when the differences in susceptibility are clarified.

The equipment, used for the assays in paper I, was standardised in order to be able to compare the results obtained between the various performers of the tests. This need arose from the fact that the different chemical treatment agents are known to differentially attach to various surfaces materials (Helgesen and Horsberg, 2013). The relatively simple bioassay protocol was designed to reduce the bias due to the fact that different persons are performing the assays. In the evaluation of the 24-hour bioassays in paper I, all parasites unattached to the bottle wall at the time of evaluation were regarded as immobilized or dead. Given that some parasites were observed swimming free in the solution when the assay was evaluated, this may have resulted in an overestimation of dead parasites. In paper II, the parasites were given a “second chance” to prove that they were still alive. However given that the control group mortality in paper I was low, misclassification was not regarded as a major issue.

Treatments might be performed in several different ways and their effect is therefore difficult to predict. The concentration of the treatment chemical may differ due to intentional increase in dose or unintentional increase or decrease in the dose as a result of miscalculation of the treatment volume. The latter may occur as it is difficult to correctly calculate the volume of an enclosed tarpaulin (Treasurer et al., 2000b). Treatment time may also be increased in an attempt to increase treatment efficacy. Simultaneous employment of several treatment agents in a treatment makes it impossible for bioassays, developed for one substance, to predict the treatment efficacy. Deltamethrin bioassays were considered suitable for use in the detection of cypermethrin resistance. This is due to the fact that both are type 2 pyrethroids with voltage gated sodium channels as their target sites (reviewed in Bloomquist, 1996). Sevatdal (2005) however found some differences in the EC_{50} -ratios (EC_{50} -values/recommended dose) for these substances, in bioassays performed on salmon lice from strains with moderately reduced sensitivity. The relationship between deltamethrin bioassays and cypermethrin treatments should therefore be taken into consideration in a validation process.

The final aspect of the validation process between bioassays and field treatment efficacies is the evaluation of the efficacy. As salmon lice are not evenly distributed amongst the salmon in a fish

farm, the counting of lice on a limited number of fish pre- and post-treatment may not necessarily give the correct treatment efficacy (Treasurer and Pope, 2000). Furthermore the chalimus stages are difficult to find on the fish and this may lead to incorrect and unreliable counts. Re-infestation of salmon lice larvae following the treatment can also influence the reported result. The development of the surviving parasites between the pre-treatment and post-treatment counting will also add uncertainty to the final result.

A two dose bioassay with 30 minutes exposure to pyrethroids to determine field resistance was suggested, but not tested, by Sevatdal (2005). A fixed dose approach was also chosen for a study on EMB susceptibility in salmon lice in Canada conducted by Whyte et al. (2013). Both seasonal and spatial differences in susceptibility were detected in the Canadian study. Variation between genders and instars was also observed. In the paper by Whyte et al. (2013) it was however never claimed that their bioassay could differentiate between sensitive and resistant parasites. The bioassay protocol, developed in paper I, was considered simpler and better compared to the traditional bioassays. It was also regarded as sufficiently well validated, based on the small-scale treatment trials in paper I, for surveillance studies. This protocol was therefore chosen as the basis for the bioassay protocol in a nationwide surveillance program for salmon lice resistance in Norway. The survey was performed for the Norwegian Food Safety Authority. In total, 145 tests at 62 salmon farming sites were carried out by local fish health services in order to detect spatial differences in deltamethrin, azamethiphos and EMB susceptibility between *L. salmonis* in Norway in 2013 (Grøntvedt et al., 2014). The program was continued in 2014 where 230 tests were carried out (Grøntvedt et al., 2015). The assays used in this surveillance program allowed for good documentation of the spatial differences in sensitivity. However the validation against treatment results is a subject for further research.

The aim of developing a simpler bioassay protocol for resistance testing of salmon lice may be seen as achieved with respect to the chemotherapeutants deltamethrin, azamethiphos and emamectin benzoate. Protocols have been developed based on paper I both to detect spatial differences in sensitivity in salmon lice along the Norwegian coast as well as to determine deltamethrin resistance in *C. rogercresseyi* in Chile (Grøntvedt et al., 2014; Grøntvedt et al., 2015; Helgesen et al., 2014). Further studies are however needed in order to develop the protocol into a tool which can predict field treatment efficacy as well as determining if the results from deltamethrin bioassays may be used to predict the efficacy of cypermethrin treatments.

Aim 2: To develop bioassay protocols suitable for resistance testing of salmon lice towards hydrogen peroxide and to apply these to selected salmon louse strains

The hydrogen peroxide (H₂O₂) bioassays described in paper II could be seen as a continuation of paper I. Reports of reduced efficacy of hydrogen peroxide treatments from the field created a need for the development of a resistance detection tool. This tool was needed in order to determine if it was resistant parasites which were causing this reduced treatment efficacy (Denholm et al., 2002). H₂O₂ resistance had been previously reported in salmon lice more than ten years ago in Scotland (Treasurer et al., 2000c). In that particular study, small-scale treatments were applied in order to test for resistance. Resistance detection using small-scale treatments is however suboptimal. This is due to the fact that this method is expensive, requires special facilities and is undesirable with respect to animal welfare. H₂O₂ bioassays, performed on salmon lice detached from fish, had been previously used when the effect of hydrogen peroxide on salmon lice was first described (Bruno and Raynard,

1994; Johnson et al., 1993; Treasurer and Grant, 1997). Given that bioassays in these studies could be applied to determine the natural tolerance level of H₂O₂ in salmon lice, bioassays were also assumed to be suitable tools in the detection of resistance.

The results of the bioassays had to be immediately evaluated post-exposure. This was due to the fact that the salmon lice studied in paper II recovered from the effect of H₂O₂ in a non-dose dependent manner if transferred to fresh sea water. The recovery of the parasites, primarily knocked out by H₂O₂, has been previously reported (Bruno and Raynard, 1994; Hodneland et al., 1993; Johnson et al., 1993; Treasurer and Grant, 1997). Treasurer and Grant (1997) also demonstrated the importance of standardizing H₂O₂ bioassays with regards to temperature. This is due to the fact that H₂O₂ is more toxic to salmon lice at higher temperatures. The differences in H₂O₂ susceptibility between the instars and genders of salmon lice are not fully understood. However the results from the south west of Norway, presented in the results section of this thesis, suggest that adult males are less sensitive than pre-adult II females. The same difference in susceptibility was observed when results from a field treatment, of presumably sensitive parasites, were evaluated by Treasurer and Grant (1997). As the bioassays in paper II were performed on a non-allocated number of pre-adults from both genders and adult males, these differences may have influenced the results. The main conclusions, regarding the sensitivity level of the different strains in paper II, were however not believed to be influenced by these differences. This is due to the large difference in EC₅₀-values observed. In order to further develop bioassays into a tool to predict treatment results, the difference in susceptibility between instars and genders must be taken into consideration.

The bioassay with 24-hours exposure to H₂O₂ was developed in a manner similar to the bioassays described in paper I. The reason behind this was that use of the same protocol on all salmon lice bioassays would be highly beneficial. By using this bioassay protocol the difference between the sensitive and the resistant salmon louse strain was still present, but somewhat reduced compared to the results from the 30-minute bioassay. The 24-hour hydrogen peroxide bioassay was therefore implemented in the Norwegian surveillance program for salmon lice resistance in 2014 (Grøntvedt et al., 2015).

H₂O₂ resistance in salmon lice, described in paper II in the salmon louse strain Ls V, was the first report of H₂O₂ resistant salmon lice from Norway. Later the 2014 Norwegian resistance surveillance program detected H₂O₂ resistance in the south-western part of Norway in addition to Mid-Norway (Grøntvedt et al., 2015). Paper II also gave the first report of the heritability of H₂O₂ resistance in salmon lice as the resistance level (EC₅₀-values) remained at the same elevated level from the F0 generation to the F1 generation. This was despite the fact that the F1 generation had never been exposed to H₂O₂. The discovery of H₂O₂ resistance completes the array of resistance found in Norwegian salmon lice towards chemical treatment agents effective against all mobile instars (Grøntvedt et al., 2014; Sevatdal and Horsberg, 2003). If farmed salmon are infested with multiple resistant salmon lice showing full field resistance towards pyrethroids, azamethiphos, EMB and H₂O₂, the benzoylureas are the only effective chemical treatment agents the salmon farmer is left with. These agents are however inefficient against adult parasites in addition of being associated with environmental disadvantages (Branson et al., 2000; Samuelsen et al., 2014).

Due to the H₂O₂ resistance found in Ls V-F1, one of the resistance mechanisms proposed in the study by Treasurer et al. (2000) is regarded as less likely: That the resistance is caused by pre-exposure to

low concentrations of H₂O₂. The preliminary results described in the results section of this thesis, however renders another theory suggested in the same paper more probable. This second theory suggests that resistance is caused by the presence of the detoxifying enzyme catalase. In our unpublished study Ls V showed a higher catalase activity and increased expression of catalase in comparison to Ls A. This coincides with findings from mammalian cells, bacteria and fungi, where increased catalase is seen to result in H₂O₂ resistance. (Amin and Olson, 1968; Nakamura et al., 2012; Spitz et al., 1992). The rate of resistance development may be enhanced by the fact that many salmon lice recovered after first being knocked out by H₂O₂. This theory is based on the fact that stress is known to boost the development of resistance in bacteria and mammalian cells (reviewed in Gressel, 2011).

Given that suitable bioassay protocols for resistance testing of salmon lice towards hydrogen peroxide were developed, it may be surmised that aim 2 was achieved. Both the 30-minute and the 24-hour bioassays were able to differentiate between the different salmon louse strains with regards to their sensitivity towards H₂O₂. Similar to the bioassays described in paper I, there is still some missing knowledge and therefore a need for further research. These bioassays need improved validation against treatment efficacy data before they can be established as field H₂O₂ resistance detection tools.

Aim 3: To elucidate the molecular mechanism behind azamethiphos resistance in salmon lice and to develop a rapid high-throughput resistance assay to detect this mechanism.

Organophosphate resistant salmon lice were found in the early 90s in Scotland and Ireland (Jones et al., 1992; Tully and McFadden, 2000). These two studies looked at the effect of the organophosphate dichlorvos. Another Scottish study reported resistance towards the currently applied organophosphate; azamethiphos (Roth et al., 1996). From Norway, studies of azamethiphos resistance were first published in paper I and in the 2013 resistance survey (Grøntvedt et al., 2014). However has organophosphate resistance been described as a problem in Norway since the early 90s (Denholm et al., 2002; Fallang et al., 2004). All of these aforementioned studies identified organophosphate resistance using treatment efficacy evaluations or results from bioassays. Although bioassays were improved as an organophosphate resistance detection method in paper I, they are attached with confounding factors, they are costly and not fully validated to predict field resistance. Molecular methods for resistance testing are therefore preferred based on their high precisions, specification and ability to detect resistance in individual salmon lice. The development of a high-throughput *in vitro* resistance assay using such molecular methods, to detect organophosphate resistance in salmon lice, would therefore be highly beneficial.

In a study by Fallang et al. (2004) on the mechanism for organophosphate resistance in salmon lice, differences in the inhibition curve of acetylcholinesterase (AChE), was examined using two protocols. In the first protocol different concentrations of azamethiphos were added to tissue homogenates and the incubation time was fixed before analysis. In a number of the samples, the AChE-activity was not completely inhibited by azamethiphos. In the second protocol, a fixed azamethiphos concentration and different incubation times was used. Of 28 lice, the AChE activity was almost completely inhibited (less than 10 % remaining activity) in 18 samples, while the remaining 10 maintained a measurable residual activity of the enzyme. The two hypotheses proposed in the paper by Fallang et al. (2004) to explain this finding were based on the existence of a modification in

the AChE gene or the existence of more than one enzyme. Both suggestions have been demonstrated to be correct, but only the first is the actual resistance mechanism. Kaur et al. (2015) found two different AChE genes (*ace1a* and *ace1b*) in *L. salmonis* and in paper III a mutation (*Phe362Tyr*) was found in *ace1a*, whereas a nonsynonymous single nucleotide polymorphism (*Ile433Thr*) was identified in *ace1b*. It was however only the mutation in *ace1a*, *Phe362Tyr*, that was associated with salmon lice resistance.

The mutation in *ace1a* is situated in the acyl pocket of the active gorge of the enzyme, in a highly conserved area of the gene, where mutations in organophosphate resistant arthropods have been previously determined (Alon et al., 2008; Nabeshima et al., 2004). The 3D-modelling of the gene showed that the *Phe362Tyr* mutation narrows the entrance of the gorge and thereby possibly hinders azamethiphos of reaching the catalytic triad, while the relatively smaller acetylcholine still is allowed access. The frequency of the mutation in the different studied salmon louse strains was associated with their exposure history to azamethiphos and with results from bioassays and small-scale treatments.

The enzymatic assay used in the second study in the paper by Fallang et al. (2004), could unfortunately not be reproduced. The cause of this non-repeatability is unclear, but a small pilot study demonstrated high protease activity in salmon lice (data not shown). One possible theory may therefore be that the AChE activity is gradually depleted after homogenisation of the samples. Thus, another protocol was chosen. The enzymatic studies in paper III were conducted within a few minutes after preparation of the samples, to avoid denaturation of the enzymes by proteases. These studies demonstrated residual activity in both sensitive and resistant parasites. This activity was however significantly higher in resistant lice. The residual AChE activity seen in the resistant lice, following inhibition with azamethiphos, was believed to be sufficient to keep these parasites alive during a treatment.

In paper III the *Phe362Tyr* mutation was also found in organophosphate resistant salmon lice from Norway, sampled in 1998, whereas none of the sensitive samples from 1998 carried the mutation. The mutation was therefore assumed to be the cause of the organophosphate resistance seen in Norway in the 90s (Fallang et al., 2004). Since the mutation most probably has survived in the population between the years 2000 and 2007 in Norway, in the absence of selection pressure from organophosphates, since this chemical group was not applied for treatments (Helgesen et al., 2014), the cost of being resistant cannot be extensive. This finding supports the theory by Shi et al. (2004) that resistance-causing mutations can survive in a population as heterozygotes for a very long period of time, even in the absence of chemotherapeutants (Shi et al., 2004). This is due to the fact that the fitness cost associated with being heterozygous for the mutation is thought to be lower than for homozygous individuals.

The success of this mutation arises to a certain degree from the partial dominance of the resistant allele. Evidence of this partial dominance of the R-allele was shown in the small-scale treatment trial designed to detect the survival rate of the different genotypes. In this trial 56 % of the heterozygous individuals survived. When an allele is rare it almost only occurs in heterozygote individuals (Georghiou and Taylor, 1977). This was the case in paper III for Ls G (4 % RS, 0 % RR). If Ls G was subjected to azamethiphos treatments, during the early generations the susceptibility of these RS individuals would have determined the rate of the evolution towards resistance.

The *Phe362Tyr* mutation is a molecular mechanism for azamethiphos resistance in salmon lice. This mutation has been determined in all examined salmon lice populations displaying reduced sensitivity towards organophosphates. Thus, it is a major resistance factor, but whether it is the only mechanism or if other mechanisms also play a role remains to be determined. High-throughput assays for the rapid diagnosis of the mutation have been developed (TaqMan assay and HRM). However increased knowledge of the survival rates, of the three different genotypes, SS, RS and RR, in field treatments, is necessary in order to be able to predict treatment efficacy. It may therefore be surmised that the aim to elucidate the molecular mechanism behind azamethiphos resistance in salmon lice and develop a rapid high-throughput assay for screening of this mechanism could be seen as nearly accomplished.

Aim 4: To elucidate the molecular mechanism for pyrethroid resistance in salmon lice and to develop a rapid high-throughput resistance assay to detect this mechanism.

Resistance towards pyrethroids in salmon lice has been seen on both sides of the Atlantic Ocean as well as in *C. rogercresseyi* in Chile (Helgesen et al., 2014; Sevatdal et al., 2005a; Whyte et al., 2014). In these studies resistance was detected using different bioassay protocols. Given that pyrethroids are extremely hydrophobic, performing bioassays with these substances is difficult as they prefer to attach to surfaces as opposed to remaining dissolved in water (reviewed in Albaseer et al., 2011). Detecting the molecular mechanisms for pyrethroid resistance in salmon lice and subsequently developing a high-throughput screening assay would therefore greatly simplify pyrethroid resistance testing.

Two studies have previously investigated the mechanisms for pyrethroid resistance in salmon lice. Evidence of monooxygenase mediated detoxification as a cause of resistance was provided by one of these studies, while the other study detected a mutation in the voltage gated sodium channel (Na_v) gene (Fallang et al., 2005; Sevatdal et al., 2005b). Mutations in Na_v genes are also known to cause pyrethroid resistance in several other arthropods (reviewed in Rinkevich et al., 2013). These genes were therefore chosen as the study targets for paper IV. However no mutations were detected in the predominantly expressed Na_v gene in *L. salmonis*. This applies to all the pyrethroid resistant parasites included in the study and includes the mutation detected by Fallang et al. (2005). Given that the salmon lice in paper IV were selected for pyrethroid resistance on an individual basis while the lice in the study by Fallang et al. (2005) were selected on a population level, based on treatment and bioassay results, the results from paper IV were assumed to give a better account of the situation.

Typical features of Na_v genes were described in the three genes identified in the salmon louse genome to be the putative Na_v genes of *L. salmonis*: $Na_v1.1$, $Na_v1.2$ and $Na_v1.3$. Differences in expression between the genes were determined in a qPCR study. The identified predominant expression of $Na_v1.1$, in comparison to the other two genes, was in accordance with the results from the transcriptome analysis available in LiceBase (<https://licebase.org/>). The qPCR study did not show any differences in the expression of the three Na_v genes between the resistant and sensitive strains of salmon lice. Given that only five parasites from each strain and only one sensitive strain were included in this study, some uncertainty surrounds this result. Furthermore, the resistant parasites had been exposed to deltamethrin, when they were individually selected for resistance, at slightly various points in time prior to collection, while the sensitive lice had not been exposed. This

difference may have influenced the expression of sodium channel genes, if the expression was induced by the treatments and had not returned to its pre-treatment level.

Typical Nav gene features were described from the three genes identified in paper IV and expression of the genes was detected. However, no studies were performed on the distribution of the Na_v RNA in the lice, on the actual protein expression level and body location, or of the function of the genes. The localization of the RNA of the three different sodium channels in the salmon louse could have been determined by applying in situ hybridization technique (Harrison et al., 1973). A study of the protein expression level of the different Na_v channels could have been performed using western blot (Renart et al., 1979) and the body location of the proteins could have been determined with immunohistochemistry as was done for the LsYAP protein by Dalvin et al. (2009). Finally the function of the three genes could have been determined using *xenopus* oocytes in electrophysiological studies (Gundersen et al., 1983).

The three gene sequences from Na_v1.1, included in the sequence analysis in paper IV, were chosen due to the fact that they include homologous regions where mutations associated with pyrethroid resistance have been found in other arthropod species (Rinkevich et al., 2013). However, not all regions of the gene, containing homologous sequences where mutations have been found in other arthropods, were included in the study (Wang et al., 2002). The large amount of heterogeneity, detected in the three discovered SNPs in Na_v1.1, indicated though no concurrent presence of a resistance mutation. Resistant individuals would be expected to belong to a specific haplotype, as was seen for EMB resistant salmon lice (Besnier et al., 2014). 95 % of Na_v1.1 was sequenced in five of the resistant lice without detecting any mutations. This is described in the results section of this thesis. These findings do not provide evidence for mutations in Na_v1.1 as a cause of pyrethroid resistance.

The absence of mutations in Na_v1.1 does however not rule out mutations in any of the other Na_v genes as a cause of pyrethroid resistance. Mutations in Na_v1.2 or Na_v1.3 were though regarded as an unlikely cause of pyrethroid resistance. This is due to the fact that compared to the aforementioned genes; Na_v1.1 was overexpressed and subsequently believed to be the predominant Na_v gene in salmon lice. The presence of three Na_v channels in salmon lice may provide an explanation as to why mutations in these genes do not cause pyrethroid resistance. If all three channels are essential for the survival of the parasite, a mutation resulting in pyrethroid insensitivity in one of the channels would have little effect. The parasite would still die after a pyrethroid treatment since the function of the other two channels would be fatally damaged. The concurrent presence of mutations in several Na_v genes has been seen in the tetrodotoxin resistant snake, *Thamnophis sirtalis* (McGlothlin et al., 2014). This was explained by the simultaneous selection pressure exerted on all channel genes.

Given that the molecular mechanism for pyrethroid resistance in salmon lice was not clarified in paper IV, no rapid high-throughput resistance assay could be developed. The pyrethroids' target sites, i.e. the voltage gated sodium channel genes, were however identified and subsequently characterized in salmon lice. A sequence study revealed no mutations in the principal Na_v gene. Further research on pyrethroid resistance should therefore be targeted at other possible resistance mechanisms.

Overall discussion

Resistant salmon- or sea lice are a common concern in nearly all salmonid producing countries (Aaen et al., 2015). Pesticide resistance also poses problems for farmers wanting to protect their crop or farmed animals from parasite attacks or infestation, as well as for people trying to combat vectors for human diseases (reviewed in Tabashnik et al., 2014). As long as chemical treatment agents are necessary weapons in the battle against these parasitic species, their developed resistance is something that must be anticipated and subsequently dealt with. Prior to performing an individual chemical treatment, establishing the resistance status of the parasites is important in order to predict the treatment efficacy. For salmon lice this can now be achieved by the use of the resistance detection methods from paper I, II and III. Inefficient treatments may be avoided if resistance testing of salmon lice is performed prior to treatment, thereby reducing the amount of chemicals used. This will subsequently be advantageous for the fish, the environment and the fish farmer.

In order to avoid resistance from developing or to slow down its development, an overall resistance management strategy is required (Phillips et al., 1989). This strategy should include a plan for optimizing the use of the different chemical treatment agents, a plan for the use of the alternatives to pesticides and a plan on how best to introduce new treatment agents (reviewed in Denholm and Rowland, 1992). A resistance management strategy must be a common tool for as large an area as required in order to avoid different strategies working against each other. The salmon lice integrated pest management (IPM) strategy is targeted at gaining control of the salmon lice (Brooks, 2009). Although the IPM strategy takes resistance into consideration in order to achieve this goal, a dedicated resistance management strategy for salmon lice would be beneficial as it would target the resistance situation especially.

The resistance detection tools developed in paper I, II and III could be implemented in a resistance management strategy both to describe the current resistance situation and to evaluate the effect of measures taken to slow the speed of resistance development. The usage data on anti-salmon lice agents compared to the total salmonid production indicates that salmon farmers are trying to increase the treatment efficacy by increasing the frequency of treatments and/or the concentrations applied (Helgesen et al., 2014). The tactic of trying to overpower resistance is known from resistance management in other parasitic species (Denholm and Rowland, 1992). Whether this method is fruitful in salmon lice could be decided on if resistance tests were implemented as part of the management strategy.

As the high-throughput resistance detection tool from paper III is more accurate than the bioassays in detecting resistance at an individual level, further effort should be put into finding the molecular mechanisms behind all types of salmon lice resistance. The work presented in paper IV has given indications that metabolic resistance should be an increased area of research with regards to pyrethroid resistance. Studies on resistance mechanisms require well-characterized salmon louse strains. This could be accomplished using the bioassays developed in paper I and II. Bioassays have already been applied to characterize salmon louse strains with regards to their chemical tolerance in several of the papers which have studied the mechanisms for resistance (Carmichael et al., 2013; Heumann et al., 2014; Sutherland et al., 2014)

Results from the present work have been adopted by the Norwegian authorities. In the 2013 national surveillance program for resistance in salmon lice, the protocol applied used methods and equipment described in paper I (Grøntvedt et al., 2014). That year, a total of 145 bioassays were conducted through the program. The new knowledge of the existence of hydrogen peroxide resistance and the newly developed molecular assay for organophosphate resistance was incorporated in the 2014 version of the surveillance program (Grøntvedt et al., 2015). In 2014, 230 bioassays and 2 molecular assays were conducted. Furthermore, many of the local fish health services along the Norwegian coast regularly monitor the sensitivity situation at their customer's sites and use the protocol from the surveillance program to carry out this task. The results from the protocol are employed when deciding which treatments agents to apply.

The papers included in this thesis have described parts of the situation regarding salmon lice resistance in Norway. Paper I was the first to describe a multidrug resistant strain of salmon lice, Ls B, which was resistant towards both pyrethroids and EMB. Paper I was also the first to characterize salmon louse strains with resistance towards emamectin benzoate and azamethiphos in Norway, although the existence of such resistance had been reported previously (Fallang et al., 2004; Horsberg, 2012). Paper II gave the first report of resistance towards hydrogen peroxide. Pyrethroid resistance had been documented earlier (Sevatdal et al., 2005a; Sevatdal and Horsberg, 2000; Sevatdal and Horsberg, 2003). However, Paper IV showed that deltamethrin resistance was widespread along the Norwegian coast. The methods developed in paper I, II and III have been well received by the authorities and local fish health services. Its implementation in a nationwide survey has detected widespread resistance towards deltamethrin, EMB and azamethiphos, as well as some areas with hydrogen peroxide resistance, in Norway (Grøntvedt et al., 2014; Grøntvedt et al., 2015).

CONCLUSION

In conclusion, the aims for this work have, to a large extent, been achieved. The results presented in paper I, II and III provide tools for the detection of salmon lice resistance towards the commonly used chemotherapeutants deltamethrin, azamethiphos, emamectin benzoate and hydrogen peroxide. Simplified bioassay protocols have been developed for all of these treatment agents, while a high-throughput assay has also been developed to detect azamethiphos resistance. The latter test was based on the detection of a mutation found in one of the acetylcholinesterase genes in salmon lice. This mutation had been previously proven to cause azamethiphos resistance. The first account of hydrogen peroxide resistance in Norway was described in paper II using the developed bioassay protocol. The three voltage gated sodium channel genes in *L. salmonis* were identified and described in paper IV. The predominantly expressed gene was subsequently screened for mutations in pyrethroid resistant parasites, however no mutation was detected. Future research on pyrethroid resistance should therefore be targeted elsewhere.

FUTURE PERSPECTIVES

Today, salmon lice are amongst the greatest concerns of the salmon farming industry. This parasite is one of the factors hindering salmonid production growth. As long as the salmon farming industry is dependent on chemical treatments to control the salmon lice levels, resistance towards these treatment agents will be a constant concern. Development of novel treatment agents will help to delay resistance. This is due to the fact that rotation between drugs with different modes of action

reduces the selection pressure on each resistance mechanism (Denholm and Rowland, 1992). Resistance will however still develop and so far, in the race between resistance development and the development of novel treatment agents, the lice have caught up with the pharmaceutical industry. An example of this is the use of H₂O₂ which has increased in Norway in recent years. H₂O₂ is being used against the emerging salmon disease amoebic gill disease in addition to in combatting salmon lice (Adams et al., 2012; Norwegian Institute of public Health, 2015). From 2013 to 2014 the use of H₂O₂ was almost four-folded, resulting in an expectation that the strength and distribution of H₂O₂ resistance in salmon lice will increase.

New treatment agents will require the development of new bioassay protocols in order to be able to detect resistance. This also includes the currently available benzoylureas, where only preliminary bioassay studies have been performed (Sevatdal, 2005). With regards to bioassays, an area of missing knowledge is the validation of the bioassays with respect to field treatment efficacies. Today bioassays may detect resistance, but they are insufficiently validated in order to give correct estimates of field treatment efficacies.

The mechanisms behind resistance in salmon lice are so far only partially understood. The cause of emamectin benzoate resistance has not been fully elucidated, despite the fact that several papers have been published on this subject. The mechanism for organophosphate resistance has been discovered in Norway, but whether or not this mechanism is universal is currently unknown. Whether or not organophosphate resistance has spread through the Atlantic Ocean in the same manner as EMB resistance may also be clarified (Besnier et al., 2014). With regards to the mechanisms behind pyrethroids and H₂O₂ resistance, a substantial amount of work needs to be performed in order to fully understand these mechanisms.

When the molecular mechanisms for resistance have been established, they can be further developed into high-throughput resistance assays. As for the bioassays, the molecular method to detect organophosphate resistance and other resistance mechanisms yet to be discovered, also need to be validated towards field treatment efficacy.

Knowledge of resistance mechanisms may also prove useful when developing new treatment chemicals, in order to avoid these chemicals from being combated by already existing resistance mechanisms. If the resistance mechanism is a type of metabolic resistance, the possibility of adding synergists to the treatment protocol, which inhibits the mechanism, should be investigated. Knowledge of resistance mechanisms is also an invaluable tool for validating models predicting the development or spread of resistance. Such models can subsequently be used to predict the effect of various anti-salmon lice interventions on resistance and by doing so improve the resistance management strategy.

Knowledge of the *Phe362Tyr* mutation and how its frequency has increased amongst azamethiphos treated parasites, in comparison to its frequency amongst the novel salmon louse strains, can also be applied to determine the origin of salmon lice on wild salmonids. Salmon lice with the mutation will have originated from salmon farms. Knowing the origin of the salmon lice found on wild salmonids is important in the debate of the effect of salmon farming on wild salmonids.

If resistance is associated with fitness cost in salmon lice is presently not fully understood. Knowledge of this area is however important in order to assess the effect of refugia for sensitive

parasites and area following as part of a resistance management strategy. It has been shown that greater resistance comes with greater fitness costs in *Helicoverpa armigera* (Cao et al., 2014). If this is also the case for *L. salmonis*, low fitness cost may be expected in salmon lice. This is due to relatively low levels of resistance as seen in the differences between bioassay EC₅₀-values of sensitive and resistant strains (up to 31 fold difference found in paper I). Insects have in contrast been found to be several hundred folds resistant (Ahmad et al., 2006).

The distribution of resistance along the coast is to a certain extent known in Norway through the resistance surveillance program of the Norwegian Food Safety Authority (Grøntvedt et al., 2014; Grøntvedt et al., 2015). The same type of knowledge would be beneficial to gain in the rest of the salmonid producing world. This would give a picture of the severity and the development of resistance in salmon lice. This knowledge could in turn be used by both authorities and by fish farmers in planning the future of salmonid aquaculture.

Specific resistance management strategies for salmon lice should be developed and resistance testing with bioassays or *in vitro* tests targeted at specific resistance mechanisms should be implemented. Resistance testing is however only a small part of the bigger picture in gaining control of the salmon lice. Optimizing the management strategies in individual farms and in areas are also important methods to achieve this aim. Even if new anti-parasitic drugs are developed, the main contribution to gain control of this parasite must come from the non-chemical methods; both by developing new methods, but even more importantly by implementing the already existing methods in an optimal manner.

REFERENCES

- Aaen, S.M., Aunsmo, A., Horsberg, T.E., 2014. Impact of hydrogen peroxide on hatching ability of egg strings from salmon lice (*Lepeophtheirus salmonis*) in a field treatment and in a laboratory study with ascending concentrations. *Aquaculture* 422, 167-171.
- Aaen, S.M., Helgesen, K.O., Bakke, M.J., Kaur, K., Horsberg, T.E., 2015. Drug resistance in sea lice: a threat to salmonid aquaculture. *Trends Parasitol* 31, 72-81.
- Adams, M.B., Crosbie, P.B.B., Nowak, B.F., 2012. Preliminary success using hydrogen peroxide to treat Atlantic salmon, *Salmo salar* L., affected with experimentally induced amoebic gill disease (AGD). *J Fish Dis* 35, 839-848.
- Ahmad, M., Denholm, I., Bromilow, R.H., 2006. Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid-resistant strains of *Helicoverpa armigera* from China and Pakistan. *Pest Manag Sci* 62, 805-810.
- Ahmad, M., Sayyed, A.H., Saleem, M.A., Ahmad, M., 2008. Evidence for field evolved resistance to newer insecticides in *Spodoptera litura* (Lepidoptera: Noctuidae) from Pakistan. *Crop Prot* 27, 1367-1372.
- Albaseer, S.S., Rao, R.N., Swamy, Y.V., Mukkanti, K., 2011. Analytical artifacts, sample handling and preservation methods of environmental samples of synthetic pyrethroids. *Trends Analyt Chem* 30, 1771-1780.
- Alon, M., Alon, F., Nauen, R., Morin, S., 2008. Organophosphates' resistance in the B-biotype of *Bemisia tabaci* (Hemiptera: Aleyrodidae) is associated with a point mutation in an ace1-type acetylcholinesterase and overexpression of carboxylesterase. *Insect Biochem Mol Biol* 38, 940-949.
- Anonym, 2014. The fish farmers believe in mechanical delousing (in Norwegian: Oppdretterne tror på mekanisk luseløsning). ilaks.no. Internet: <http://www.ilaks.no/oppdretterne-tror-pa-mekanisk-luselosning/> Accessed: 12.03.15.
- Amin, V.M., Olson, N.F., 1968. Influence of catalase activity on resistance of coagulase-positive staphylococci to hydrogen peroxide. *Appl Microbiol* 16, 267-270.
- Arena, J.P., Liu, K.K., Paress, P.S., Frazier, E.G., Cully, D.F., Mrozik, H., Schaeffer, J.M., 1995. The mechanism of action of avermectins in *Caenorhabditis elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. *J Parasitol* 81, 286-294.
- Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22, 195-201.
- Bass, C., Field, L.M., 2011. Gene amplification and insecticide resistance. *Pest Manag Sci* 67, 886-890.
- Baud, O., Greene, A.E., Li, J.R., Wang, H., Volpe, J.J., Rosenberg, P.A., 2004. Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *J Neurosci* 24, 1531-1540.
- Beattie, M., 2009. Industry Update on Pesticide Usage. In: Integrated pest management. Sea lice treatments, application requirements and systemic use workshop. New Brunswick Salmon Growers

Association, St. Andrews, pp 9-10. Internet: http://0101.nccdn.net/1_5/038/248/08d/Integrated-Pest-Management-Workshop-Dec-10-2009.pdf Accessed: 12.03.15.

Berge, A., 2015. Same results from all thermic deloused sites (in Norwegian: Samme prosenter på alle lokaliteter vi har kjørt termisk avlusning). ilaks.no. Internet: <http://www.ilaks.no/samme-prosenter-pa-alle-lokaliteter-vi-har-kjort-termisk-avlusning/> Accessed: 12.03.15.

Besnier, F., Kent, M., Skern-Mauritzen, R., Lien, S., Malde, K., Edvardsen, R.B., Taylor, S., Ljungfeldt, L.E., Nilsen, F., Glover, K.A., 2014. Human-induced evolution caught in action: SNP-array reveals rapid amphi-atlantic spread of pesticide resistance in the salmon ectoparasite *Lepeophtheirus salmonis*. BMC Genomics 15.

Bloomquist, J.R., 1996. Ion channels as targets for insecticides. Annu Rev Entomol 41, 163-190.

Bowers, J.M., Mustafa, A., Speare, D.J., Conboy, G.A., Brimacombe, M., Sims, D.E., Burka, J.F., 2000. The physiological response of Atlantic salmon, *Salmo salar* L., to a single experimental challenge with sea lice, *Lepeophtheirus salmonis*. J Fish Dis 23, 165-172.

Bowers, J.M., Speare, D.J., Burka, J.F., 2002. The effects of hydrogen peroxide on the stress response of Atlantic Salmon (*Salmo salar*). J Vet Pharmacol Ther 25, 311-313.

Boxaspen, K., 2006. A review of the biology and genetics of sea lice. Ices J Mar Sci 63, 1304-1316.

Braden, L.M., Barker, D.E., Koop, B.F., Jones, S.R.M., 2012. Comparative defense-associated responses in salmon skin elicited by the ectoparasite *Lepeophtheirus salmonis*. Comp Biochem Physiol Part D Genomics Proteomics 7, 100-109.

Brandal, P.O., Egidius, E., Romslo, I., 1976. Host blood - major food component for parasitic copepod *Lepeophtheirus salmonis* Kroyeri, 1838 (Crustacea-Caligidae). Nor J Zool 24, 341-343.

Branson, E.J., Ronsberg, S.S., Ritchie, G., 2000. Efficacy of teflubenzuron (Calicide®) for the treatment of sea lice, *Lepeophtheirus salmonis* (Krøyer 1838), infestations of farmed Atlantic salmon (*Salmo salar* L.). Aquac Res 31, 861-867.

Bravo, S., 2003. Sea lice in Chilean salmon farms. Bull Eur Assn Fish P 23, 197-200.

Bristow, G.A., Berland, B., 1991. The effect of long term, low level *Eubothrium* sp (Cestoda: Pseudophyllidea) infection on growth in farmed salmon (*Salmo salar* L.). Aquaculture 98, 325-330.

Brooks, K.M., 2009. Considerations in developing an integrated pest management programme for control of sea lice on farmed salmon in Pacific Canada. J Fish Dis 32, 59-73.

Bruno, D.W., Raynard, R.S., 1994. Studies on the use of hydrogen peroxide as a method for the control of sea lice on Atlantic salmon. Aquacult Int 2, 10-18.

Burrige, L., Lyons, M., Wong, D., MacKeigan, K., VanGeest, J., 2014. The acute lethality of three anti-sea lice formulations: AlphaMax®, Salmosan®, and Interlox®Paramove™50 to lobster and shrimp. Aquaculture 420, 180-186.

Cao, G., Feng, H., Guo, F., Wu, K., Li, X., Liang, G., Desneux, N., 2014. Quantitative analysis of fitness costs associated with the development of resistance to the Bt toxin Cry1Ac in *Helicoverpa armigera*. Sci Rep 4, 5629.

Carmichael, S.N., Bron, J.E., Taggart, J.B., Ireland, J.H., Bekaert, M., Burgess, S.T., Skuce, P.J., Nisbet, A.J., Gharbi, K., Sturm, A., 2013. Salmon lice (*Lepeophtheirus salmonis*) showing varying emamectin benzoate susceptibilities differ in neuronal acetylcholine receptor and GABA-gated chloride channel mRNA expression. BMC Genomics 14, 408.

Centers for Disease Control and Prevention, 2015. CDC Bottle Bioassays. Internet: http://www.cdc.gov/parasites/education_training/lab/bottlebioassay.html Accessed: 12.03.15.

Charlier, J., Morgan, E., Rinaldi, L., van Dijk, J., Demeler, J., Høglund, J., Hertzberg, H., Van Ranst, B., Hendrickx, G., Vercruyse, J., Kenyon, F., 2014. Practices to optimise gastrointestinal nematode control on sheep, goat and cattle farms in Europe using targeted (selective) treatments. Vet Rec 175, 250-255.

Chavez-Mardones, J., Gallardo-Escárate, C., 2014. Deltamethrin (AlphaMax™) reveals modulation of genes related to oxidative stress in the ectoparasite *Caligus rogercresseyi*: Implications on delousing drug effectiveness. Aquaculture 433, 421-429.

Corbel, V., N'Guessan, R., 2013. Distribution, mechanisms, impact and management of insecticide resistance in malaria vectors: A pragmatic review. In: Manguin, S. (Ed.), Anopheles mosquitoes - New insights into malaria vectors. InTech.

Cornejo, I., 2014. Identification and functional expression of a glutamate- and avermectin-gated chloride channel from *Caligus rogercresseyi*, a Southern Hemisphere sea louse affecting farmed fish. PLoS Pathog 10, e1004402.

Costello, M.J., 1996. Development and future of cleaner-fish technology and other biological control techniques in fish farming. In: Sayer, M.D.J., Treasurer, J.W., Costello, M.J. (Eds.), Wrasse biology and use in aquaculture. Blackwell Science Ltd, London, pp. 171-184.

Costello, M.J., 2009. The global economic cost of sea lice to the salmonid farming industry. J Fish Dis 32, 115-118.

Dalvin, S., Frost, P., Biering, E., Hamre, L.A., Eichner, C., Krossøy, B., Nilsen, F., 2009. Functional characterisation of the maternal yolk-associated protein (LsYAP) utilising systemic RNA interference in the salmon louse (*Lepeophtheirus salmonis*) (Crustacea: Copepoda). Int J Parasitol 39, 1407-1415.

Denholm, I., Devine, G.J., Horsberg, T.E., Sevatdal, S., Fallang, A., Nolan, D.V., Powell, R., 2002. Analysis and management of resistance to chemotherapeutants in salmon lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae). Pest Manag Sci 58, 528-536.

Denholm, I., Rowland, M.W., 1992. Tactics for managing pesticide resistance in arthropods: theory and practice. Annu Rev Entomol 37, 91-112.

Duce, I.R., Bhandal, N.S., Scott, R.H., Norris, T.M., 1995. Effects of ivermectin on gamma-aminobutyric acid and glutamate-gated chloride conductance in arthropod skeletal muscle. In: Clark, J.M. (Ed.), Molecular action of insecticides on ion channels. American Chemical Society, Washington, DC, pp. 251-263.

Eichner, C., Hamre, L.A., Nilsen, F., 2015. Instar growth and molt increments in *Lepeophtheirus salmonis* (Copepoda: Caligidae) chalimus larvae. Parasitol Int 64, 86-96.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7, 88-90.

- Ernst, W., Jackman, P., Doe, K., Page, F., Julien, G., Mackay, K., Sutherland, T., 2001. Dispersion and toxicity to non-target aquatic organisms of pesticides used to treat sea lice on salmon in net pen enclosures. *Mar Pollut Bull* 42, 433-444.
- Espedal, P.G., Glover, K.A., Horsberg, T.E., Nilsen, F., 2013. Emamectin benzoate resistance and fitness in laboratory reared salmon lice (*Lepeophtheirus salmonis*). *Aquaculture* 416, 111-118.
- Falk, K., Namork, E., Rimstad, E., Mjaaland, S., Dannevig, B.H., 1997. Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). *J Virol* 71, 9016-9023.
- Fallang, A., Denholm, I., Horsberg, T.E., Williamson, M.S., 2005. Novel point mutation in the sodium channel gene of pyrethroid-resistant sea lice *Lepeophtheirus salmonis* (Crustacea: Copepoda). *Dis Aquat Organ* 65, 129-136.
- Fallang, A., Ramsay, J.M., Sevatdal, S., Burka, J.F., Jewess, P., Hammell, K.L., Horsberg, T.E., 2004. Evidence for occurrence of an organophosphate-resistant type of acetylcholinesterase in strains of sea lice (*Lepeophtheirus salmonis* Krøyer). *Pest Manag Sci* 60, 1163-1170.
- Finnegan, M., Linley, E., Denyer, S.P., McDonnell, G., Simons, C., Maillard, J.Y., 2010. Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms. *Antimicrob Chemother* 65, 2108-2115.
- Food and Agriculture Organization of the United Nations, 2015. Global aquaculture production 1950-2012. Internet: <http://www.fao.org/fishery/statistics/global-aquaculture-production/en> Accessed: 12.03.15.
- Frost, P., Nilsen, F., Hamre, L.A., 2007. Novel sea lice vaccine. World Intellectual Property Organization, Geneva. International Publication Number WO/2007/039599 A1.
- Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. *Environ Health Perspect* 87, 245-254.
- Georghiou, G.P., Taylor, C.E., 1977. Genetic and biological influences in evolution of insecticide resistance. *J Econ Entomol* 70, 319-323.
- Glover, K.A., Aasmundstad, T., Nilsen, F., Storset, A., Skaala, O., 2005. Variation of Atlantic salmon families (*Salmo salar* L.) in susceptibility to the sea lice *Lepeophtheirus salmonis* and *Caligus elongatus*. *Aquaculture* 245, 19-30.
- Grant, A.N., 2002. Medicines for sea lice. *Pest Manag Sci* 58, 521-527.
- Gressel, J., 2011. Low pesticide rates may hasten the evolution of resistance by increasing mutation frequencies. *Pest Manag Sci* 67, 253-257.
- Grimnes, A., Jakobsen, P.J., 1996. The physiological effects of salmon lice infection on post-smolt of Atlantic salmon. *J Fish Biol* 48, 1179-1194.
- Grøntvedt, R.N., Jansen, P., Horsberg, T.E., Helgesen, K.O., Tarpai, A., 2014. The surveillance programme for resistance to chemotherapeutants in salmon lice (*Lepeophtheirus salmonis*) in Norway 2013. Norwegian Veterinary Institute, Oslo. Internet: <http://www.vetinst.no/Publikasjoner/Overvaakingsprogrammer-OK/2013/The-surveillance->

programme-for-resistance-to-chemotherapeutants-in-L.-salmonis-in-Norway-2013 Accessed: 12.03.15.

Grøntvedt, R.N., Jansen, P., Horsberg, T.E., Helgesen, K.O., Tarpai, A., 2015. The surveillance programme for resistance to chemotherapeutants in salmon lice (*Lepeophtheirus salmonis*) in Norway 2014. Norwegian Veterinary Institute, Oslo. Internet: <http://www.vetinst.no/Publikasjoner/Overvaakingsprogrammer-OK/2014/The-surveillance-programme-for-resistance-to-chemotherapeutants-in-salmon-lice-Lepeophtheirus-salmonis-in-Norway-2014> Accessed: 25.03.15.

Grøntvedt, R.N., Kristoffersen, A.B., 2015. Permanent skirts may reduce the infestation of salmon lice - analysis of field collected data (in Norwegian: Permaskjørt kan redusere påslag av lakselus – analyse av feltdata – Delrapport Permaskjørt A5). Norwegian Veterinary Institute, Oslo. Internet: <http://www.vetinst.no/Publikasjoner/Rapportserie/Rapportserie-2015/Permaskjoert-kan-reducere-paaslag-av-lakselus-analyse-av-feltdata-Delrapport-Permaskjoert-A5> Accessed: 12.03.15.

Gudding, R., Lillehaug, A., Evensen, Ø., 2010. Vaccination of fish (in Norwegian: Vaksinasjon av fisk). In: Gudding, R. (Ed.), Vaccination of animals (in Norwegian: Vaksinasjon av dyr). Scandinavian Veterinary Press, Oslo, pp. 144-181.

Gundersen, C.B., Miledi, R., Parker, I., 1983. Voltage-operated channels induced by foreign messenger-RNA in *Xenopus* oocytes. Proc R Soc Lond B Biol Sci 220, 131-140.

Hamre, L.A., Eichner, C., Caipang, C.M.A., Dalvin, S.T., Bron, J.E., Nilsen, F., Boxshall, G., Skern-Mauritzen, R., 2013. The salmon louse *Lepeophtheirus salmonis* (Copepoda: Caligidae) life cycle has only two chalimus stages. PLOS ONE 8.

Hamre, L.A., Glover, K.A., Nilsen, F., 2009. Establishment and characterisation of salmon louse (*Lepeophtheirus salmonis* (Krøyer 1837)) laboratory strains. Parasitol Int 58, 451-460.

Hanssen, T.M., Lie, E., 2014. New technology can prevent salmon lice. The Research Council of Norway. Internet: http://www.forskningsradet.no/prognett-havbruk/Nyheter/New_technology_can_prevent_salmon_lice/1253996529392/p1226994216948 Accessed: 12.03.15.

Harel, M., Kryger, G., Rosenberry, T.L., Mallender, W.D., Lewis, T., Fletcher, R.J., Guss, J.M., Silman, I., Sussman, J.L., 2000. Three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and of its complexes with two potent inhibitors. Protein Sci 9, 1063-1072.

Harrison, P.R., Conkie, D., Paul, J., Jones, K., 1973. Localization of cellular globin messenger-RNA by in-situ hybridization to complementary DNA. Febs Lett 32, 109-112.

Hart, J.L., Thacker, J.R., Braidwood, J.C., Fraser, N.R., Matthews, J.E., 1997. Novel cypermethrin formulation for the control of sea lice on salmon (*Salmo salar*). Vet Rec 140, 179-181.

Hastie, L.C., Wallace, C., Birkett, M.A., Douglas, A., Jones, O., Mordue, L., Ritchie, G., Pickett, J.A., Webster, J.L., Bowman, A.S., 2013. Prevalence and infection intensity of sea lice (*Lepeophtheirus salmonis*) on Atlantic salmon (*Salmo salar*) host is reduced by the non-host compound 2-aminoacetophenone. Aquaculture 410-411, 179-183.

Haya, K., 1989. Toxicity of pyrethroid insecticides to fish. Environ Toxicol Chem 8, 381-391.

- Helgesen, K.O., Bravo, S., Sevatdal, S., Mendoza, J., Horsberg, T.E., 2014. Deltamethrin resistance in the sea louse *Caligus rogercresseyi* (Boxhall and Bravo) in Chile: bioassay results and usage data for antiparasitic agents with references to Norwegian conditions. *J Fish Dis* 37, 877-890.
- Helgesen, K.O., Horsberg, T.E., 2013. Influence of different materials on the concentration of delousing agents in sea water during bioassays. *J Fish Dis* 36, 529-532.
- Heuch, P.A., Nordhagen, J.R., Schram, T.A., 2000. Egg production in the salmon louse (*Lepeophtheirus salmonis* (Krøyer)) in relation to origin and water temperature. *Aquac Res* 31, 805-814.
- Heuch, P.A., Parsons, A., Boxaspen, K., 1995. Diel vertical migration: A possible host-finding mechanism in salmon louse (*Lepeophtheirus salmonis*) Copepodids? *Can J Fish Aquat Sci* 52, 681-689.
- Heumann, J., Carmichael, S., Bron, J.E., Tildesley, A., Sturm, A., 2012. Molecular cloning and characterisation of a novel P-glycoprotein in the salmon louse *Lepeophtheirus salmonis*. *Comp Biochem Physiol C Toxicol Pharmacol* 155, 198-205.
- Heumann, J., Carmichael, S.N., Bron, J.E., Sturm, A., 2014. Isolation and characterisation of four partial cDNA sequences encoding multidrug resistance-associated proteins (MRPs) in the salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837). *Aquaculture* 424, 207-214.
- Hodneland, K., Nylund, A., Nilsen, F., Midttun, B., 1993. The effect of Nuvan, azamethiphos and hydrogen peroxide on salmon lice (*Lepeophtheirus salmonis*). *Bull Eur Assn Fish P* 13, 203-206.
- Horsberg, T.E., 2012. Avermectin use in aquaculture. *Curr Pharm Biotechnol* 13, 1095-1102.
- Hotelier, T., Negre, V., Marchot, P., Chatonnet, A., 2010. Insecticide resistance through mutations in cholinesterases or carboxylesterases: data mining in the ESTHER database. *J Pestic Sci* 35, 315-320.
- Igboeli, O.O., Burka, J.F., Fast, M.D., 2013. Sea lice population and sex differences in P-glycoprotein expression and emamectin benzoate resistance on salmon farms in the Bay of Fundy, New Brunswick, Canada. *Pest Manag Sci* 70, 905-914.
- Igboeli, O.O., Fast, M.D., Heumann, J., Burka, J.F., 2012. Role of P-glycoprotein in emamectin benzoate (SLICE (R)) resistance in sea lice, *Lepeophtheirus salmonis*. *Aquaculture* 344, 40-47.
- Imsland, A.K., Reynolds, P., Eliassen, G., Hangstad, T.A., Foss, A., Vikingstad, E., Elvegard, T.A., 2014. The use of lumpfish (*Cyclopterus lumpus* L) to control sea lice (*Lepeophtheirus salmonis* Krøyer) infestations in intensively farmed Atlantic salmon (*Salmo salar* L). *Aquaculture* 424, 18-23.
- Iversen, M., Finstad, B., McKinley, R.S., Eliassen, R.A., Carlsen, K.T., Evjen, T., 2005. Stress responses in Atlantic salmon (*Salmo salar* L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. *Aquaculture* 243, 373-382.
- Jackson, D., Cotter, D., Newell, J., McEvoy, S., O'Donohoe, P., Kane, F., McDermott, T., Kelly, S., Drumm, A., 2013. Impact of *Lepeophtheirus salmonis* infestations on migrating Atlantic salmon, *Salmo salar* L., smolts at eight locations in Ireland with an analysis of lice-induced marine mortality. *J Fish Dis* 36, 273-281.
- Jakob, E., Barker, D.E., Garver, K.A., 2011. Vector potential of the salmon louse *Lepeophtheirus salmonis* in the transmission of infectious haematopoietic necrosis virus (IHNV). *Dis Aquat Organ* 97, 155-165.

- Johnson, S.C., Albright, L.J., 1992. Comparative susceptibility and histopathology of the response of naive Atlantic, chinook and coho salmon to experimental-infection with *Lepeophtheirus salmonis* (Copepoda, Caligidae). *Dis Aquat Organ* 14, 179-193.
- Johnson, S.C., Constible, J.M., Richard, J., 1993. Laboratory investigations on the efficacy of hydrogen peroxide against the salmon louse *Lepeophtheirus salmonis* and its toxicological and histopathological effects on Atlantic salmon *Salmo salar* and Chinook salmon *Oncorhynchus tshawytscha*. *Dis Aquat Organ* 17, 197-204.
- Jones, M.W., Sommerville, C., Wootten, R., 1992. Reduced sensitivity of the salmon louse, *Lepeophtheirus salmonis*, to the organophosphate dichlorvos. *J Fish Dis* 15, 197-202.
- Jones, P.G., Hammell, K.L., Dohoo, I.R., Revie, C.W., 2012. Effectiveness of emamectin benzoate for treatment of *Lepeophtheirus salmonis* on farmed Atlantic salmon *Salmo salar* in the Bay of Fundy, Canada. *Dis Aquat Organ* 102, 53-64.
- Jonsdottir, H., Bron, J.E., Wootten, R., Turnbull, J.F., 1992. The histopathology associated with the preadult and adult stages of *Lepeophtheirus salmonis* on the Atlantic salmon, *Salmo salar* L. *J Fish Dis* 15, 521-527.
- Kabata, Z., 1974. Mouth and mode of feeding of Caligidae (Copepoda), parasites of fishes, as determined by light and scanning electron-microscopy. *J Fish Res Board Can* 31, 1583-1588.
- Kaur, K., Bakke, M.J., Nilsen, F., Horsberg, T.E., 2015. Identification and molecular characterization of two acetylcholinesterases from the salmon louse, *Lepeophtheirus salmonis*. *PLOS ONE*, in press.
- Krkošek, M., Ford, J.S., Morton, A., Lele, S., Myers, R.A., Lewis, M.A., 2007. Declining wild salmon populations in relation to parasites from farm salmon. *Science* 318, 1772-1775.
- Krkošek, M., Revie, C.W., Finstad, B., Todd, C.D., 2014. Comment on Jackson et al. 'Impact of *Lepeophtheirus salmonis* infestations on migrating Atlantic salmon, *Salmo salar* L., smolts at eight locations in Ireland with an analysis of lice-induced marine mortality'. *J Fish Dis* 37, 415-417.
- Kruger, M., Van Rensburg, J.B.J., Van den Berg, J., 2009. Perspective on the development of stem borer resistance to Bt maize and refuge compliance at the Vaalharts irrigation scheme in South Africa. *Crop Prot* 28, 684-689.
- Kutti, T., Hansen, P.K., Ervik, A., Hoisaeter, T., Johannessen, P., 2007. Effects of organic effluents from a salmon farm on a fjord system. II. Temporal and spatial patterns in infauna community composition. *Aquaculture* 262, 355-366.
- Kwon, D.H., Song, D.Y., Kang, S., Ahn, J.J., Lee, J.H., Choi, B.R., Lee, S.W., Kim, J.H., Lee, S.H., 2010. Residual contact vial bioassay for the on-site detection of acaricide resistance in the two-spotted spider mite. *J Asia Pac Entomol* 13, 333-337.
- Langford, K.H., Oxnevad, S., Schoyen, M., Thomas, K.V., 2014. Do antiparasitic medicines used in aquaculture pose a risk to the Norwegian aquatic environment? *Environmental Science & Technology* 48, 7774-7780.
- Leclercq, E., Davie, A., Emigaud, H., 2014. Delousing efficiency of farmed ballan wrasse (*Labrus bergylta*) against *Lepeophtheirus salmonis* infecting Atlantic salmon (*Salmo salar*) post-smolts. *Pest Manag Sci* 70, 1274-1282.

- Lees, F., Baillie, M., Gettinby, G., Revie, C.W., 2008. The efficacy of emamectin benzoate against infestations of *Lepeophtheirus salmonis* on farmed Atlantic salmon (*Salmo salar* L) in Scotland, 2002-2006. PLOS ONE 3, e1549.
- Løvoll, M., Wiik-Nielsen, J., Grove, S., Wiik-Nielsen, C.R., Kristoffersen, A.B., Faller, R., Poppe, T., Jung, J., Pedamallu, C.S., Nederbragt, A.J., Meyerson, M., Rimstad, E., Tengs, T., 2010. A novel totivirus and piscine reovirus (PRV) in Atlantic salmon (*Salmo salar*) with cardiomyopathy syndrome (CMS). Virol J 7.
- McClure, C.A., Hammell, K.L., Dohoo, I.R., 2005. Risk factors for outbreaks of infectious salmon anemia in farmed Atlantic salmon, *Salmo salar*. Prev Vet Med 72, 263-280.
- McGlothlin, J.W., Chuckalovcak, J.P., Janes, D.E., Edwards, S.V., Feldman, C.R., Brodie, E.D., Pfrender, M.E., Brodie, E.D., 2014. Parallel evolution of tetrodotoxin resistance in three voltage-gated sodium channel genes in the garter snake *Thamnophis sirtalis*. Mol Biol Evol 31, 2836-2846.
- Merzendorfer, H., 2013. Chitin synthesis inhibitors: old molecules and new developments. Insect Sci 20, 121-138.
- Midtlyng, P.J., Grave, K., Horsberg, T.E., 2011. What has been done to minimize the use of antibacterial and antiparasitic drugs in Norwegian aquaculture? Aquac Res 42, 28-34.
- Mordue, A.J., Pike, A.W., 2002. Salmon farming: towards an integrated pest management strategy for sea lice. Pest Manag Sci 58, 513-514.
- Mork, J., 1991. One-generation effects of farmed fish immigration on the genetic differentiation of wild Atlantic salmon in Norway. Aquaculture 98, 267-276.
- Müller, P., Donnelly, M.J., Ranson, H., 2007. Transcription profiling of a recently colonised pyrethroid resistant *Anopheles gambiae* strain from Ghana. BMC Genomics 8.
- Mutoh, N., Nakagawa, C.W., Yamada, K., 1999. The role of catalase in hydrogen peroxide resistance in fission yeast *Schizosaccharomyces pombe*. Can J Microbiol 45, 125-129.
- Nabeshima, T., Mori, A., Kozaki, T., Iwata, Y., Hidoh, O., Harada, S., Kasai, S., Severson, D.W., Kono, Y., Tomita, T., 2004. An amino acid substitution attributable to insecticide-insensitivity of acetylcholinesterase in a Japanese encephalitis vector mosquito, *Culex tritaeniorhynchus*. Biochem Biophys Res Commun 313, 794-801.
- Nakamura, K., Kanno, T., Mokudai, T., Iwasawa, A., Niwano, Y., Kohno, M., 2012. Microbial resistance in relation to catalase activity to oxidative stress induced by photolysis of hydrogen peroxide. Microbiol Immunol 56, 48-55.
- Nolan, D.T., Reilly, P., Bonga, S.E.W., 1999. Infection with low numbers of the sea louse *Lepeophtheirus salmonis* induces stress-related effects in postsmolt Atlantic salmon (*Salmo salar*). Can J Fish Aquat Sci 56, 947-959.
- Norwegian Institute of public Health, 2015. The usage of anti-salmon lice agents are high and still increasing (in Norwegian: Forbruket av lakselusmidler er høyt og øker fortsatt). Internet: http://www.fhi.no/eway/default.aspx?pid=239&trg=Content_6496&Main_6157=6263:0:25,6201&MainContent_6263=6496:0:25,6205&Content_6496=6178:114175:25,6205:0:6562:1:::0:0 Accessed: 12.03.15.

- Nowak, B., Hayward, C., Gonzalez, L., Bott, N., Lester, R.I., 2011. Sea lice infections of salmonids farmed in Australia. *Aquaculture* 320, 171-177.
- Nylund, A., Wallace, C., Hovland, T., 1993. The possible role of *Lepeophtheirus salmonis* (Krøyer) in the transmission of infectious salmon anaemia. In: Boxshall, G., Defaye, D. (eds.) *Pathogens of wild and farmed fish: sea lice*. Ellis Horwood Ltd, London, p. 367-373.
- Palacios, G., Lovoll, M., Tengs, T., Hornig, M., Hutchison, S., Hui, J., Kongtorp, R.T., Savji, N., Bussetti, A.V., Solovyov, A., Kristoffersen, A.B., Celone, C., Street, C., Trifonov, V., Hirschberg, D.L., Rabadan, R., Egholm, M., Rimstad, E., Lipkin, W., I, 2010. Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus. *PLOS ONE* 5.
- Phillips, J.R., Graves, J.B., Luttrell, R.G., 1989. Insecticide resistance management: Relationship to integrated pest management. *Pestic Sci* 27, 459-464.
- Pohle, G., Frost, B., Findlay, R., 2001. Assessment of regional benthic impact of salmon mariculture within the Letang Inlet, Bay of Fundy. *Ices J Mar Sci* 58, 417-426.
- Purcell, S.L., Friend, S.E., Covelto, J.M., Donkin, A., Groman, D.B., Poley, J., Fast, M.D., 2013. CpG inclusion in feed reduces sea lice, *Lepeophtheirus salmonis*, numbers following re-infection. *J Fish Dis* 36, 229-240.
- Ramsden, N., 2014. First sea lice shot with a laser; costs just €4m. *undercurrentnews*. Internet: <http://www.undercurrentnews.com/2014/06/19/first-sea-lice-shot-with-a-laser-costs-just-e4m/> Accessed: 12.03.15.
- Ranson, H., N'Guessan, R., Lines, J., Moiroux, N., Nkuni, Z., Corbel, V., 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol* 27, 91-98.
- Renart, J., Reiser, J., Stark, G.R., 1979. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci USA* 76, 3116-3120.
- Riddell, B.E., Beamish, R.J., Richards, L.J., Candy, J.R., 2008. Comment on "Declining wild salmon populations in relation to parasites from farm salmon". *Science* 322, 1790B.
- Rinkevich, F.D., Du, Y.Z., Dong, K., 2013. Diversity and convergence of sodium channel mutations involved in resistance to pyrethroids. *Pestic Biochem Physiol* 106, 93-100.
- Robertson, J.L., Preisler, H.K., Ng, S.S., Hickie, L.A., Gelernter, W.D., 1995. Natural variation: a complicating factor in bioassays with chemical and microbial pesticides. *J Econ Entomol* 88, 1-10.
- Robertson, J.L., Savin, N.E., Preisler, H.K., Russel, R.M., 2007. *Bioassays in arthropods*. CMC Press, New York.
- Ross, N.W., Johnsen, S.C., Fast, M.D., Ewart, K.V., 2006. Recombinant vaccines against caligid copepods (sea lice) and antigen sequences thereof. World Intellectual Property Organization, Geneva. International Publication Number WO/2006/010265
- Roth, M., 2000. The availability and use of chemotherapeutic sea lice control products. *Contrib Zool* 69, 109-118.

- Roth, M., Richards, R.H., Dobson, D.P., Rae, G.H., 1996. Field trials on the efficacy of the organophosphorus compound azamethiphos for the control of sea lice (Copepoda: Caligidae) infestations of farmed Atlantic salmon (*Salmo salar*). *Aquaculture* 140, 217-239.
- Roth, M., Richards, R.H., Sommerville, C., 1993. Current practices in the chemotherapeutic control of sea lice infestations in aquaculture: a review. *J Fish Dis* 16, 1-26.
- Rozas, M., Enriquez, R., 2014. Piscirickettsiosis and *Piscirickettsia salmonis* in fish: a review. *J Fish Dis* 37, 163-188.
- Ruane, N.M., Jones, S.R.M., 2013. Amoebic gill disease (AGD) of farmed Atlantic salmon (*Salmo salar* L.). ICES Identification Leaflets for Diseases and Parasites of Fish and Shellfish. Internet: <http://www.ices.dk/sites/pub/Publication%20Reports/Disease%20Leaflets/Sheet%20no.%2060.pdf> Accessed: 12.03.15.
- Rust, M.K., Denholm, I., Dryden, M.W., Payne, P., Blagburn, B.L., Jacobs, D.E., Mencke, N., Schroeder, I., Vaughn, M., Mehlhorn, H., Hinkle, N.C., Williamson, M., 2005. Determining a diagnostic dose for imidacloprid susceptibility testing of field-collected isolates of cat fleas (Siphonaptera: Pulicidae). *J Med Entomol* 42, 631-636.
- Saksida, S., 2006. Infectious haematopoietic necrosis epidemic (2001 to 2003) in farmed Atlantic salmon *Salmo salar* in British Columbia. *Dis Aquat Organ* 72, 213-223.
- Saksida, S.M., Morrison, D., Revie, C.W., 2010. The efficacy of emamectin benzoate against infestations of sea lice, *Lepeophtheirus salmonis*, on farmed Atlantic salmon, *Salmo salar* L., in British Columbia. *J Fish Dis* 33, 913-917.
- Samuelsen, O.B., Lunestad, B.T., Farestveit, E., Grefsrud, E.S., Hannisdal, R., Holmelid, B., Tjensvoll, T., Agnalt, A.L., 2014. Mortality and deformities in European lobster (*Homarus gammarus*) juveniles exposed to the anti-parasitic drug teflubenzuron. *Aquat Toxicol* 149, 8-15.
- Sawicki, R.M., Denholm, I., 1987. Management of resistance to pesticides in cotton pests. *Trop Pest Manage* 33, 262-272.
- Sevatdal, S., 2005. Sea lice resistance to chemotherapeutants: Bioassay as diagnostic tools for determination of sensitivity patterns in sea lice (*Lepeophtheirus salmonis* Krøyer). Norwegian School of Veterinary Science, Oslo. Thesis for the degree of *Philosophiae Doctor*.
- Sevatdal, S., Copley, L., Wallace, C., Jackson, D., Horsberg, T.E., 2005a. Monitoring of the sensitivity of sea lice (*Lepeophtheirus salmonis*) to pyrethroids in Norway, Ireland and Scotland using bioassays and probit modelling. *Aquaculture* 244, 19-27.
- Sevatdal, S., Fallang, A., Ingebrigtsen, K., Horsberg, T.E., 2005b. Monooxygenase mediated pyrethroid detoxification in sea lice (*Lepeophtheirus salmonis*). *Pest Manag Sci* 61, 772-778.
- Sevatdal, S., Horsberg, T.E., 2000. The identification of pyrethroid resistance in sea lice (in Norwegian: Kartlegging av pyretroidresistens hos lakselus). *Norsk fiskeoppdrett* 12, 34-35.
- Sevatdal, S., Horsberg, T.E., 2003. Determination of reduced sensitivity in sea lice (*Lepeophtheirus salmonis* Krøyer) against the pyrethroid deltamethrin using bioassays and probit modelling. *Aquaculture* 218, 21-31.

- Shi, M.A., Lougarre, A., Alies, C., Fremaux, I., Tang, Z.H., Stojan, J., Fournier, D., 2004. Acetylcholinesterase alterations reveal the fitness cost of mutations conferring insecticide resistance. *BMC Evol Biol* 4.
- Sjern-Mauritzen, R., Torrissen, O., Glover, K.A., 2014. Pacific and Atlantic *Lepeophtheirus salmonis* (Krøyer, 1838) are allopatric subspecies: *Lepeophtheirus salmonis salmonis* and *L. salmonis oncorhynchi* subspecies novo. *BMC Genetics* 15.
- Soderlund, D.M., Knipple, D.C., 2003. The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochem Mol Biol* 33, 563-577.
- Spitz, D.R., Adams, D.T., Sherman, C.M., Roberts, R.J., 1992. Mechanisms of cellular resistance to hydrogen peroxide, hyperoxia, and 4-hydroxy-2-nonenal toxicity - the significance of increased catalase activity in H₂O₂-resistant fibroblasts. *Arch Biochem Biophys* 292, 221-227.
- Statistics Norway, 2014. External trade in goods, 2013, final numbers (in Norwegian: Utenrikshandel med varer, 2013, endelige tall). Internet: <http://www.ssb.no/utenriksokonomi/statistikker/muh/aar-enderlige> Accessed: 12.03.15.
- Stone, J., Sutherland, I.H., Sommerville, C.S., Richards, R.H., Varma, K.J., 1999. The efficacy of emamectin benzoate as an oral treatment of sea lice, *Lepeophtheirus salmonis* (Krøyer), infestations in Atlantic salmon, *Salmo salar* L. *J Fish Dis* 22, 261-270.
- Sutherland, B.J.G., Koczka, K.W., Yasuike, M., Jantzen, S.G., Yazawa, R., Koop, B.F., Jones, S.R.M., 2014a. Comparative transcriptomics of Atlantic *Salmo salar*, chum *Oncorhynchus keta* and pink salmon *O. gorbuscha* during infections with salmon lice *Lepeophtheirus salmonis*. *BMC Genomics* 15.
- Sutherland, B.J.G., Poley, J.D., Igboeli, O.O., Jantzen, J.R., Fast, M.D., Koop, B.F., Jones, S.R.M., 2014b. Transcriptomic responses to emamectin benzoate in Pacific and Atlantic Canada salmon lice *Lepeophtheirus salmonis* with differing levels of drug resistance. *Evol Appl* 8, 133-148.
- Tabashnik, B.E., Mota-Sanchez, D., Whalon, M.E., Hollingworth, R.M., Carriere, Y., 2014. Defining terms for proactive management of resistance to Bt crops and pesticides. *J Econ Entomol* 107, 496-507.
- Tal, Y., Schreier, H.J., Sowers, K.R., Stubblefield, J.D., Place, A.R., Zohar, Y., 2009. Environmentally sustainable land-based marine aquaculture. *Aquaculture* 286, 28-35.
- Tamura, K., Stecher, G., Peterson, D., Filipowski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30, 2725-2729.
- Thomassen, J.M., 1993. A new method for control of salmon lice. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinnereim, K. (Eds.), *Fish Farming Technology*. Balkema, Rotterdam, pp. 233-236.
- Tobback, E., Decostere, A., Hermans, K., Haesebrouck, F., Chiers, K., 2007. *Yersinia ruckeri* infections in salmonid fish. *J Fish Dis* 30, 257-268.
- Toovey, J.P.G., Lyndon, A.R., 2000. Effects of hydrogen peroxide, dichlorvos and cypermethrin on subsequent fecundity of sea lice, *Lepeophtheirus salmonis*, under fish farm conditions. *Bull Eur Assn Fish P* 20, 224-228.
- Toranzo, A.E., Magarinos, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. *Aquaculture* 246, 37-61.

- Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O.T., Nilsen, F., Horsberg, T.E., Jackson, D., 2013. Salmon lice - impact on wild salmonids and salmon aquaculture. *J Fish Dis* 36, 171-194.
- Treasurer, J.W., Grant, A., 1997. The efficacy of hydrogen peroxide for the treatment of farmed Atlantic salmon, *Salmo salar* L infested with sea lice (Copepoda: Caligidae). *Aquaculture* 148, 265-275.
- Treasurer, J.W., Grant, A., Davis, P.J., 2000a. Physical constraints of bath treatments of Atlantic salmon (*Salmo salar*) with a sea lice burden (Copepoda : Caligidae). *Contrib Zool* 69, 129-136.
- Treasurer, J.W., Pope, J.A., 2000. Selection of host sample number and design of a monitoring programme for ectoparasitic sea lice (Copepoda : Caligidae) on farmed Atlantic salmon, *Salmo salar*. *Aquaculture* 187, 247-260.
- Treasurer, J.W., Wadsworth, S., Grant, A., 2000c. Resistance of sea lice, *Lepeophtheirus salmonis* (Krøyer), to hydrogen peroxide on farmed Atlantic salmon, *Salmo salar* L. *Aquac Res* 31, 855-860.
- Tully, O., McFadden, Y., 2000. Variation in sensitivity of sea lice (*Lepeophtheirus salmonis* (Krøyer)) to dichlorvos on Irish salmon farms in 1991-92. *Aquac Res* 31, 849-854.
- Valenzuela-Muñoz, V., Nuñez-Acuña, G., Gallardo-Escárate, C., 2014. Molecular Characterization and Transcription Analysis of P-Glycoprotein Gene from the Salmon Louse *Caligus rogercresseyi*. *J Aquac Res Dev* 5, 1000236.
- Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W., Tirry, L., 2010. Acaricide resistance mechanisms in the two-spotted spider mite *Tetranychus urticae* and other important Acari: a review. *Insect Biochem Mol Biol* 40, 563-572.
- Vontas, J.G., Small, G.J., Hemingway, J., 2001. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochem J* 357, 65-72.
- Wagner, G.N., McKinley, R.S., Bjorn, P.A., Finstad, B., 2004. Short-term freshwater exposure benefits sea lice-infected Atlantic salmon. *J Fish Biol* 64, 1593-1604.
- Wang, R.W., Liu, Z.Q., Dong, K., Elzen, P.J., Pettis, J., Huang, Z.Y., 2002. Association of novel mutations in a sodium channel gene with fluvalinate resistance in the mite, *Varroa destructor*. *J Apic Res* 41, 17-25.
- Westcott, J.D., Stryhn, H., Burka, J.F., Hammell, K.L., 2008. Optimization and field use of a bioassay to monitor sea lice *Lepeophtheirus salmonis* sensitivity to emamectin benzoate. *Dis Aquat Organ* 79, 119-131.
- Weston, J.H., Welsh, M.D., McLoughlin, M.F., Todd, D., 1999. Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, *Salmo salar* L. *Virology* 256, 188-195.
- Whyte, S.K., Westcott, J.D., Elmoslemany, A., Hammell, K.L., Revie, C.W., 2013. A fixed-dose approach to conducting emamectin benzoate tolerance assessments on field-collected sea lice, *Lepeophtheirus salmonis*. *J Fish Dis* 36, 283-292.
- Whyte, S.K., Westcott, J.D., Jimenez, D., Revie, C.W., Hammell, K.L., 2014. Assessment of sea lice (*Lepeophtheirus salmonis*) management in New Brunswick, Canada using deltamethrin (AlphaMax®) through clinical field treatment and laboratory bioassay responses. *Aquaculture* 422-423, 54-62.

- Wolf, K., 1988. Infectious pancreatic necrosis. In: Wolf, K. (Ed.), Fish viruses and fish viral diseases. Cornell University Press, Ithaca, pp. 115-157.
- Wolstenholme, A.J., Kaplan, R.M., 2012. Resistance to macrocyclic lactones. *Curr Pharm Biotechnol* 13, 873-887.
- Wootten, R., Smith, J.W., Needham, E.A., 1982. Aspects of the biology of the parasitic copepods *Lepeophtheirus salmonis* and *Caligus elongatus* on farmed salmonids, and their treatment. *Proceedings of the Royal Society of Edinburgh Section B-Biological Sciences* 81, 185-197.
- World Health Organization, 1957. Seventh report of the WHO expert committee on insecticides. *World Health Organ Tech Rep Ser.*, pp. 1-32.
- World Health Organization, 1998a. Techniques to detect insecticide resistance mechanisms (field and laboratory manual). WHO/CDS/CPC/MAL/98.6. Internet: http://www.who.int/malaria/publications/atoz/who_cds_cpc_mal_98_6/en/ Accessed: 12.03.15.
- World Health Organization, 1998b. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticide on treated surfaces. Internet: <http://www.who.int/whopes/resistance/en/> Accessed: 12.03.15.
- Xu, L., Wu, M., Han, Z.J., 2013. Overexpression of multiple detoxification genes in deltamethrin resistant *Laodelphax striatellus* (Hemiptera: Delphacidae) in China. *PLOS ONE* 8.
- Young, N., Crosbie, P., Adams, M., Nowak, B., Morrison, R., 2007. *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar*). *International Journal for Parasitology* 37, 1469-1481.

ENCLOSED PAPERS I-IV

I



Single-dose field bioassay for sensitivity testing in sea lice, *Lepeophtheirus salmonis*: development of a rapid diagnostic tool

K O Helgesen and T E Horsberg

Department of Pharmacology and Toxicology, Norwegian School of Veterinary Science, Oslo, Norway

Abstract

Sea lice on farmed salmonids are often treated with chemicals. Sensitivity testing of sea lice can reduce the number of treatments by identifying substances the sea lice are susceptible to. This study describes a simpler protocol for field sensitivity testing than today's six-dose bioassay. The protocol, which uses a single dose of the delousing agents deltamethrin, azamethiphos and emamectin benzoate, was developed on four different strains of sea lice and their subsequent generations. A sensitive strain and a strain showing reduced sensitivity were identified for each chemical after performing traditional bioassays and small-scale treatments. The single doses for each chemical were established by modelling dose–response curves from 24-h bioassays on strains with differences in sensitivity. The largest difference between the lower 80% prediction interval for the sensitive strain and the upper 80% prediction interval for the strain showing reduced sensitivity was identified for each delousing agent. The concentration of the chemical and the % mortality corresponding to each of the 80% prediction intervals were subsequently established. To validate the protocol for field use, further studies on both sensitive and resistant strains of sea lice under field conditions are required.

Keywords: bioassay, *Lepeophtheirus salmonis*, resistance, sea lice, sensitivity testing, single dose.

Correspondence K O Helgesen, Department of Pharmacology and Toxicology, Norwegian School of Veterinary Science, PO box 8146 Dep, N-0033 Oslo, Norway (e-mail: kari.helgesen@nvh.no)

Introduction

Sea lice (Crustacea: Copepoda), infecting farmed salmonids, is a problem in many salmonid-producing countries. *Lepeophtheirus salmonis* (Krøyer) is the species which most commonly infects farmed salmonids in the Northern Hemisphere (as reviewed in Pike & Wadsworth 2000), while *Caligus rogercresseyi* (Boxhall & Bravo) is the most common copepod found on farmed salmonids in Chile (Carvajal, Gonzalez & George-Nascimento 1998; Boxshall & Bravo 2000; Bravo 2003). The sea lice cause stress, skin damage and osmoregulation problems (Grimnes & Jakobsen 1996; Bowers *et al.* 2000; Finstad *et al.* 2000), and the infected farmed salmonids represent a sea lice reservoir for wild salmonids (as reviewed by Costello 2009a). Different strategies have been chosen to combat sea lice, with chemical therapy being one of them. Several chemicals have been or are currently being used to combat sea lice (Grant 2002; Grave *et al.* 2004). Chemical treatment is a stressful experience for the fish (Bowers, Speare & Burka 2002), a potential threat for the surrounding wild life (as reviewed in Burrige *et al.* 2010), in addition to being an economical cost (Costello 2009b). Furthermore, chemical treatment may select for resistant parasites, thereby leaving the most resistant to form the next generation (Murray 2011).

Resistance to chemotherapeutants in sea lice has been reported from several countries in the recent years. In Scotland in 1992, for instance, Jones *et al.* reported on dichlorvos-resistant sea lice. These findings were based on bioassays initiated in sites with reduced treatment efficacy (Jones, Sommerville & Wootten 1992). An increase in

post-treatment sea lice abundance, following treatment with emamectin benzoate (EMB), was reported in Scotland between the years 2002 and 2006 (Lees *et al.* 2008). The same study indicated a regional difference in this post-treatment abundance. Treatment results and bioassays have also shown a variation in azamethiphos sensitivity in sea lice in Scotland (Roth *et al.* 1996). Evidence of resistance to hydrogen peroxide in Scotland has been reported in experimental bin treatments. Furthermore, poor efficacy in farm treatment of sea lice on a farm, previously repeatedly treated with hydrogen peroxide, was also reported (Treasurer, Wadsworth & Grant 2000). In Chile, EMB resistance was found in sea lice, *Caligus rogercresseyi*, collected from 18 different salmon farms and tested in a seven-dose bioassay (Bravo, Sevatdal & Horsberg 2008). In Norway, resistance to deltamethrin and cypermethrin in sea lice, from a farm with treatment failures, has been reported (Sevatdal & Horsberg 2003; Sevatdal *et al.* 2005). The reintroduction of azamethiphos treatment of sea lice in Norway in 2007 may have been due to treatment failure of one or more of the chemical treatment options available at that time (Midtlyng, Grave & Horsberg 2011).

An important objective in the treatment of sea lice is to limit the amount of chemicals used. To achieve this goal, high efficacy chemicals are the preferred chemical treatment option. Sea lice sensitivity testing provides an indication as to whether or not the sea lice are susceptible to the chemical. Current sensitivity testing consists of six-dose toxicological tests performed on preadult sea lice and bioassays. These bioassays are developed for deltamethrin, cypermethrin and EMB (Sevatdal & Horsberg 2003; Bravo *et al.* 2008; Westcott *et al.* 2008) but are also used for azamethiphos. These tests require 120 parasites for each substance and are relatively labour-intensive as they include several dilutions of the chemicals in addition to a cleansing step. Furthermore, different protocols are employed for each substance. Expertise is required in order to interpret these tests, as there might be difficulties deciding whether the sea lice are alive or moribund. These bioassays are developed for laboratory use on first-generation reared parasites (Sevatdal & Horsberg 2003; Bravo *et al.* 2008) or reared parasites and parasites collected from the field (Westcott *et al.* 2008), but are presently also used for field sensitivity testing.

The aim of this study was to develop a simpler field bioassay protocol for chemotherapeutant sensitivity testing of the sea louse *Lepeophtheirus salmonis*.

Materials and methods

Fish

The fish used in these studies were seawater-adapted Atlantic salmon, *Salmo salar* L. (200–1000 g), or rainbow trout, *Oncorhynchus mykiss* (Walbaum), (100–300 g). The salmon were raised at the experimental station and accustomed to sea water at least 3 months prior to the first experiment. The rainbow trout were raised at a local hatchery and brought to the experimental station and accustomed to sea water 1 month prior to the first experiment. The fish were kept in 100- to 200-L fibre glass tanks continuously supplied with sea water from 60-m depth. The temperature of the inlet water varied between 5 and 12 °C. In periods where the temperature was low, the water was heated to approximately 2 °C above the inlet temperature.

Sea lice

The sea lice used in this study were collected from four different locations in the Norwegian regions Finnmark, Nord- and Sør-Trøndelag in 2009, 2010 and 2011. The sites were chosen to provide sea lice with varying sensitivity profiles towards deltamethrin, EMB and azamethiphos. These profiles were based on frequency of treatments, the outcome of applied treatments and/or previous sensitivity tests. The sea lice strain *Lepeophtheirus salmonis* A (LS A) was collected from a site in an area previously treated with only EMB, with no treatment failures. The area is in the most northern part of Norway where sea lice resistance so far has not been reported. LS B was collected from a site reporting treatment failures after treatments with pyrethroids, EMB and azamethiphos. LS R was from a site reporting treatment failures after treatment with pyrethroids. LS H was collected from a site where azamethiphos had been used repeatedly for treatment with decreasing effect. All of the three latter were from sites in the middle part of Norway where reports of treatment failures are frequent. The sea lice were collected either as adult females with egg strings or as egg strings and subsequently transported to the laboratory in cooled sea water.

The sea lice were kept in a continuous culture using Atlantic salmon or rainbow trout as parasitic hosts. To rear a new generation of sea lice, the fish was anaesthetized with 80 mg L⁻¹ benzocaine for approximately 3 min and subsequently transferred to a tank with 25 mg L⁻¹ benzocaine. The egg strings were then manually removed from the gravid females and hatched in a fenestrated 0.5-L plastic cylinder centrally attached in a 7-L plastic container with a continuous exchange of sea water. The egg strings were incubated in the central cylinder, and after hatching, the nauplii could escape through the holes. The inlet water was filtered through a filter with pore size of 100 µm. A similar filter was attached to the outlet of the container to prevent the parasites from escaping. Air supplement through an air hose at the bottom of the central cylinder provided ventilation in addition to movement of the egg strings.

When the larvae reached the copepodid stage, the fish were infected by challenging fish with a predetermined number of copepodids. This occurred in a container containing 20 L of aerated sea water, for 15 min. When the parasites reached the preadult stages, the fish were anaesthetized as previously described and the parasites were gently removed from the fish with a forceps and subsequently used in bioassay experiments. Rainbow trout containing preadult I or II sea lice were used for small-scale treatments with deltamethrin or azamethiphos. Fish carrying sea lice expected to develop into preadult II within 3 weeks were used for small-scale treatments with EMB. All laboratory work involving fish was conducted at the Norwegian Institute of Water Research (NIVA) Marine Research Station at Drøbak, while the bioassays were set up at the Norwegian School of Veterinary Science in Oslo. It took a maximum of 4 h following the removal of the sea lice from the fish until the bioassays were initiated. In the interim period, the parasites were kept in filtered sea water at 8–12 °C. Parasites, not used in bioassays or small-scale experiments, were left on the fish for breeding of a new generation. The number of parasites per fish was adjusted to a level which caused minimum amount of skin damage to the fish (maximum 10 parasites for a 200 g fish).

Traditional bioassays

The deltamethrin bioassays were conducted according to Sevatdal & Horsberg (2003). Some modifications were made. Six concentrations in

the range 0–14 µg L⁻¹ were used for the deltamethrin bioassays. The deltamethrin stock solution was prepared by dissolving 200 µL AlphaMax (deltamethrin 10 mg mL⁻¹, Pharmaq AS) in 1000 mL sea water, while the working solution was made by dissolving 20 mL of the stock solution in 1980 mL sea water. The working solution was subsequently added to the bioassay containers in appropriate concentrations. A similar protocol was used for azamethiphos. Concentrations in the range 0–140 µg L⁻¹ were used. The stock solution was made by dissolving 5 mg azamethiphos (Prod. nr. 45331, Pestinal®) in 250 mL ethanol, while the working solution was made by dissolving 20 mL stock solution in 1980 mL water. The EMB bioassays were conducted according to Westcott *et al.* (2008), with some modifications. The six concentrations employed were in the range 0–1050 µg L⁻¹. The stock solution was made by dissolving 6 mg EMB (EMB, analytical standard, Schering-Plough) in 40 mL ethanol, and the working solution was made by dissolving 20 mL stock solution in 1980 mL sea water.

The sea lice were carefully transferred to a bucket of sea water. At the laboratory, they were placed in polystyrene boxes (Sevatdal & Horsberg 2003) and subsequently exposed to the various concentrations of deltamethrin, azamethiphos or EMB in 1-L polypropylene containers. With regard to deltamethrin, the exposure time was 30 min, 60 min for azamethiphos, and for EMB, the exposure time was 24 h. Following exposure to deltamethrin or azamethiphos, the boxes with the parasites were carefully rinsed in clean sea water and transferred to a 6-L container with sea water. The exposed parasites were incubated at 12 °C for 24 h and subsequently classified as live or moribund/dead as described by Sevatdal & Horsberg (2003).

Stability tests demonstrated that the stock solutions of azamethiphos and EMB were stable for at least 3 months after preparation (data not shown), while the deltamethrin stock solution had to be prepared immediately prior to each experiment. In all cases, the working solutions were prepared immediately prior to the experiments.

Small-scale treatment

Eight to twenty fish were used for each small-scale treatment (an overview is given in Table 1). The

Table 1 Details from small-scale treatments: the number of treated and control fish exposed to each treatment is given. Strain of sea lice and type of treatment [either in-feed treatment with emamectin benzoate (EMB) or bath treatment with deltamethrin (delt) or azamethiphos (aza)] are given in the first row. Dates of treatment and sea lice counting on treated and control fish are given in the next two rows

Strain/Treatment	LS B EMB	LS A EMB	LS A delt	LS R delt	LS B delt	LS A aza	LS H aza
Date of treatment	26 September 2011–2 October 2011	2 May 2011–8 May 2011	18 May 2011	16 September 2011	14 November 2011	14 November 2011	21 October 2011
Date of counting	17 October 2011	26 May 2011	26 May 2011	23 September 2011	21 November 2011	21 October 2011	28 October 2011
No. of fish treated	10	10	4	5	6	5	9
No. of fish control	10	10	4	6	5	5	7
Comments	Ok	Ok	Ok	Ok	Ok	Ok	*

LS, *Lepeophtheirus salmonis*.

*Indicates that one of the fish from the control group died 4 days after treatment.

bath treatments were conducted on rainbow trout infected with sea lice, held individually in glass aquaria containing 38 L of water each. The continuous water supply was sea water supplied from 60-m depth. The treatment concentrations and the treatment time used were according to the manufacturer's recommendations. A random selection was performed to divide fish into treatment and control groups. When the treatment started, the water flow was stopped to prevent dilution of the substance. The appropriate amount of the chemical to be tested was subsequently added to each tank. When the treatment time had expired, the water was drained to just above the dorsal fin of the fish, the aquarium was rapidly refilled with clean sea water, and the continuous water flow was resumed. The fish were held in the glass tanks for the next 7 days, before they were gently netted from the aquarium, anaesthetized as previously described and the number of sea lice counted.

For the deltamethrin treatments, a concentration of 2 mg per 1000 L of AlphaMax (deltamethrin 10 mg mL⁻¹, Pharmaq AS) was used. To obtain this concentration, a working solution containing 760 µL AlphaMax/L sea water was prepared in a volumetric flask. Ten millilitre working solution was subsequently added to the aquaria. Fresh working solution was made for each aquarium. Ten millilitres sea water was added to the tanks containing the control fish. The exposure time was 30 min.

A concentration of 0.1 g per 1000 L Salmosan (50% w/w azamethiphos, Fish Vet Group) was used for the azamethiphos treatments. This concentration was obtained by preparing a working solution of 760 mg Salmosan per litre fresh water (380 mg azamethiphos L⁻¹) and using 10 mL working solution per tank. 10 mL sea water was added to the tanks containing the control fish. The exposure time was 30 min.

The oral treatment with EMB was conducted on sea lice-infected salmon and trout. The product used was feed (4.5-mm pellets, Skretting) medicated with Slice (EMB 10 mg kg⁻¹, MSD). The fish were held in two groups in tanks next to each other and were not handled from the time of infection until termination of the experiments. The total weight of the fish in each tank was estimated by weighing five fish, from the same batch of fish, on the first day of treatment. The treatment started 3 weeks before the sea lice

were expected to reach the preadult II stage (Johnson & Albright 1991; Bjorn & Finstad 1998). The treatment tank and control tank were randomly selected. The treated fish were fed 0.5% of their body weight with medicated feed containing EMB (Slice) per day for 7 days (50 µg EMB per kg bodyweight per day). Uneaten medicated feed were not measured. The control fish were fed their regular feed at the same feeding rate. For the next 2 weeks, both tanks were fed their regular, non-medicated feed *ad libitum*. Two weeks after the end of the treatment period, the fish were gently netted from the tanks, anaesthetized and the number of parasites on each fish recorded.

24-h bioassays

The bioassays were conducted in 1-L glass flasks (Duran, item number 215-1786, VWR). Sea water at 12 °C was filtered through a 100 µm filter, and 1000 mL was added to each flask. Preadult sea lice were then put into the flasks. Only lice attached to the container wall or swimming in a straight line were used in the tests. The chemicals were stirred into the flasks, and the flasks were then kept at 12 °C in the dark for 24 h. The containers with sea lice were ventilated by means of an air hose from an aquarium pump connected to the flask by a 120-mm hollow needle through the lid into the water.

After 24 h, the flasks were turned upside down three times and rotated in a circle 10 times. The content was then rapidly poured out through a funnel containing a filter. 25% of the solution was subsequently returned to the flask, and the procedure was repeated. The lice on the filter and the lice remaining in the flask were counted and classified according to sex and developmental stage.

For the deltamethrin assays, concentrations in the range 0–0.8 µg L⁻¹ were used. A working solution was made by dissolving 25 µL AlphaMax in 1000 mL sea water in a polystyrene flask and subsequently diluting with clean sea water to appropriate concentrations. The working solution was used immediately after preparation. The bottle with the control group was stirred, but nothing was added.

For the azamethiphos assays, concentrations in the range 0–100 µg L⁻¹ were used. Working solutions of 5, 50 and 500 µg mL⁻¹ azamethiphos were made. A 500-µg mL⁻¹ solution was

made by dissolving 10 mg azamethiphos in 20 mL ethanol. Fifty and five microgram per millilitre solutions were made using a 10-fold dilution of the 500 µg mL⁻¹ solution with ethanol. The final concentrations in the assay flasks, which were between 0.1 and 0.5 µg L⁻¹, were obtained by adding appropriate amounts of the 5 µg mL⁻¹ working solution to the bioassay containers. Similarly, concentrations between 1 and 5 µg L⁻¹ were obtained by adding the 50-µg mL⁻¹ working solution to the flasks in appropriate amounts. Finally, appropriate amounts of the 500 µg mL⁻¹ working solution were added to the flasks to achieve concentrations between 10 and 100 µg L⁻¹. The control groups were exposed to 100 µL ethanol per litre sea water.

For the EMB assays, concentrations in the range 0–250 µg L⁻¹ were used. A working solution containing 1 mg EMB per mL ethanol was made and subsequently diluted with clean sea water. The control groups were exposed to 200 µL ethanol per litre sea water.

Statistics

For the traditional bioassays and the 24-h bioassays, EC50-values, that is the concentration where 50% of the parasites are immobilized, were calculated with 90% confidence intervals (CI). This was performed using four-parametric, nonlinear regression on dose–response data. The statistical software JMP (SAS Institute Inc.) was used to perform these calculations. Only bioassays with at least one mortality result between 0% and 100% could be modelled. For the 24-h bioassays, 80% prediction intervals (PI) were also constructed around the curves. The biggest difference, between the lower 80% PI of the most sensitive group and the upper 80% PI of the least sensitive group, was calculated. Only bioassays, both traditional and 24-h bioassays, where the mortality in the control group was lower than 20%, were included. All results from the 24-h bioassays fulfilling this criterion were pooled for each strain and chemical and used for modelling. Mortality in the control group was used to correct mortality in the other concentration groups using Schneider–Orelli's formula for both traditional and 24-h bioassays (as referred in Berndt, Meyhofer & Poehling 2004). The mean treatment efficacies in the small-scale treatments were calculated using bootstrapping with 2500 simulations. The differences (in %) of the

parasite mortalities between the treated groups and their respective control groups were calculated using the statistical software JMP. The 95% confidence intervals (CI) for efficacy were constructed using the number of fish per strain as N.

Results

Traditional bioassays performed on LS A gave EC50-values of 0.20 and 0.72 $\mu\text{g L}^{-1}$ for deltamethrin. The EC50-value was 1.73 for LS R, while bioassays on LS B gave EC50-values of 2.79 and 3.82 $\mu\text{g L}^{-1}$. This depicts a 2.4–19 times decrease in sensitivity from the susceptible to the reduced-sensitive strains, based on traditional bioassays. For azamethiphos, the EC50-value for LS A was 1.01 $\mu\text{g L}^{-1}$ in the traditional bioassay, while the values for LS H were 10.5 and 13.1 $\mu\text{g L}^{-1}$. This gave a decrease in sensitivity of 10.4–13.0, from the sensitive to the reduced-sensitive strain. For EMB, LS A had an EC50-value of 51.4 $\mu\text{g L}^{-1}$, while LS B had EC50-values of 243 and 302 $\mu\text{g L}^{-1}$. This gave a 4.7–5.9 times decrease in sensitivity from the sensitive to the reduced-sensitive strain. The results are summarized in Table 2. The mortality in the control group varied between 0% and 10%, with 0% as the median value.

Small-scale treatment with deltamethrin gave a 94.7 (95% CI, 84.2–100)% reduction in the number of sea lice in LS A, a 70.6 (59.6–81.5)% reduction in LS B and a 68.8 (53.6–84.0)% reduction in LS R. Azamethiphos treatment gave a 100% (92.8–100) reduction in sea lice abundance for LS A and a 49.8% (41.7–57.8) reduction for LS H. Following treatment with EMB, the number of sea lice was reduced by 72.6 (69.0–76.2)% for LS A and by 61.2 (56.0–66.5)% for LS B (Fig. 1). In all cases, the differences in treatment efficacy (deltamethrin, 24.1% and 25.9%; azamethiphos, 50.2%; EMB, 11.4%) were statistically significant ($P < 0.05$).

In the dose–response curves for the 24-h deltamethrin bioassays, the largest difference, between the lower 80% PI for LS A and the upper 80% PI for LS R/LS B, was 77.6%. This difference was calculated at a concentration of 0.04 $\mu\text{g L}^{-1}$. At this concentration, the lower 80% PI for LS A was 95.5% mortality, while the upper 80% PI for LS R/LS B was 17.8% mortality (Fig. 2). For azamethiphos, the greatest difference, between the lower 80% PI for LS A and upper 80% PI for LS

Table 2 Results from traditional bioassays and 24-h bioassays conducted on four different strains of sea lice. Type of chemical and strain of sea lice are given in the first column. The concentrations immobilizing 50% of the parasites (EC50) given in $\mu\text{g L}^{-1}$ with corresponding two-sided 90% confidence intervals (CI) for the traditional bioassays are given in the next three columns. The bioassays on the same strain with the same chemical are conducted 0.5–2 years apart

Strain and chemical	Traditional bioassays			24-h bioassays		
	EC50	90% CI low	90% CI high	EC50	90% CI low	90% CI high
Deltamethrin						
LS A	0.20	0.15	0.24	0.02	0.02	0.02
LS A	0.72	0.50	1.10			
LS B	2.79	1.39	151	0.38	0.24	0.54
LS B	3.82	3.41	4.48			
LS R	1.73	1.41	2.18	0.49	0.41	0.57
Azamethiphos						
LS A	1.01	*	*	0.12	0.11	0.14
LS H	10.5	7.31	61.1	3.72	2.14	11.5
LS H	13.1	5.86	132			
Emamectin benzoate						
LS A	51.4	*	*	21.5	18.2	23.7
LS B	243	127	409	167	138	199
LS B	302	*	*			

LS, *Lepeophtheirus salmonis*.

*Indicates that the CI could not be calculated as there were not enough data points giving more than 0 and <100% mortality.

For the 24-h bioassays, all results from the same strain and chemical were pooled before EC50 with 90% CI were calculated.

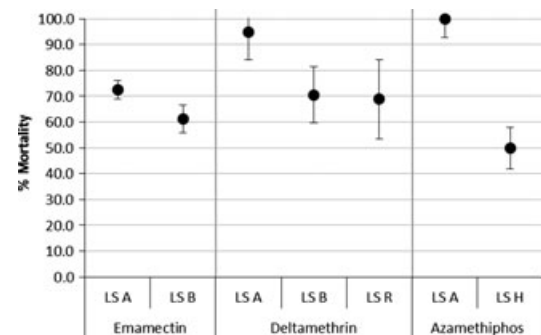


Figure 1 Results from small-scale treatment with emamectin benzoate (50 $\mu\text{g EMB}$ per kg bodyweight per day) on *Lepeophtheirus salmonis* (LS) A and LS B, deltamethrin (2 mg per 1000 L) on LS A, LS B and LS R, and azamethiphos (0.1 g per 1000 L) on LS A and LS H. The results are shown as mean treatment efficacy (black dots) with a two-sided 95% confidence interval. The number of fish in each group was between 8 and 20.

H, was 44.9%. This result was calculated at a concentration of 0.3 $\mu\text{g L}^{-1}$. The mortality at this concentration, for the lower 80% PI for LS A, was 88.7%, while for LS H the upper 80% PI

Figure 2 Twenty-four hour deltamethrin bioassay on *Lepeophtheirus salmonis* (LS) A (black line) and LS B/LS R (grey line) with 80% prediction intervals (PI). The individual points represent one group from a bioassay. The dotted line shows the biggest difference between the lower 80% PI of LS A and the upper 80% PI of LS B/LS R.

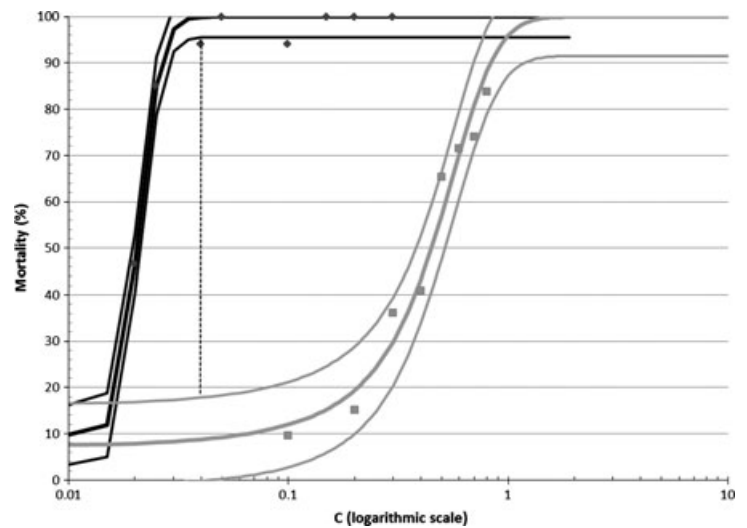
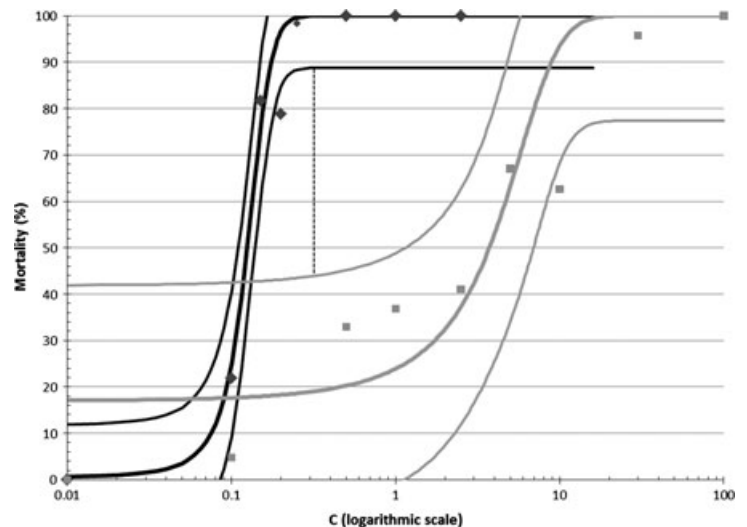


Figure 3 Twenty-four hour azamethiphos bioassay on *Lepeophtheirus salmonis* (LS) A (black line) and LS H (grey line) with 80% prediction intervals (PI). The individual points represent one group from a bioassay. The dotted line shows the biggest difference between the lower 80% PI of LS A and the upper 80% PI of LS H.



mortality was 43.8% (Fig. 3). With respect to EMB, the largest difference was 63.5% and this difference was calculated at a concentration of $47 \mu\text{g L}^{-1}$. At this concentration, the mortality for the lower 80% PI for LS A was 93.2% and the upper 80% PI for LS B was 29.7% (Fig. 4). The mortality in the control group varied between 0% and 18%, with 5.5% as the median value.

Discussion

This study describes a simpler method for sensitivity testing of sea lice than traditional six-dose bioassays (Sevatdal & Horsberg 2003; Westcott *et al.* 2008). The concentration, which distinguished

best between a sensitive strain of sea lice and a strain showing reduced sensitivity towards deltamethrin, azamethiphos and EMB, was identified. The accuracy of one single-dose bioassay was also described.

The sea lice strains chosen for the development of the single-dose bioassay represented both sensitive and reduced-sensitive strains of sea lice for each delousing agent. Optimally, several strains of sea lice from both sensitive and reduced-sensitive populations for each delousing agent should have been included in the development of the bioassay, because there are differences in the sensitivity within both groups (Roth *et al.* 1996; Sevatdal *et al.* 2005; Westcott *et al.* 2008). When this was

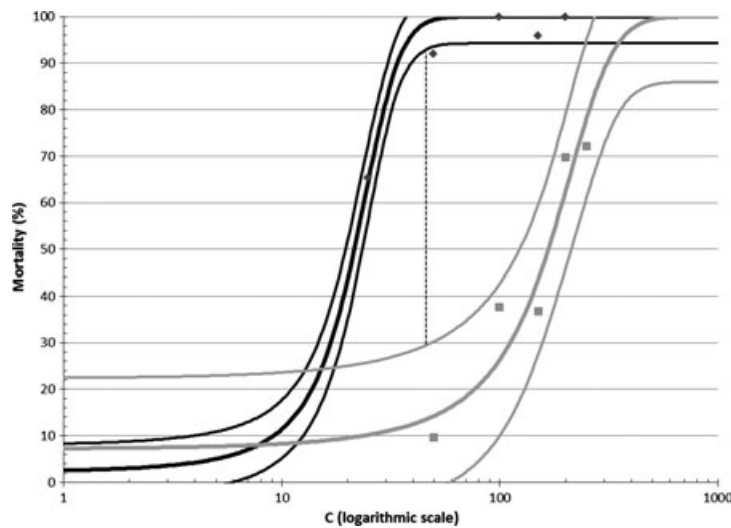


Figure 4 Twenty-four hour emamectin benzoate bioassay on *Lepeophtheirus salmonis* (LS) A (black line) and LS B (grey line) with 80% prediction intervals (PI). The individual points represent one group from a bioassay. The dotted line shows the biggest difference between the lower 80% PI of LS A and the upper 80% PI of LS B.

not possible due to practical conditions, a further step in the development of the bioassay is necessary to validate it for field use. Whether the chosen sea lice strains were good representatives from the groups of sensitive and reduced-sensitive strains of sea lice is not possible to say because there is no existing survey of the resistance status of *Lepeophtheirus salmonis* (Krøyer) in any country. Also, there is no existing documented gradient system connecting the results from the traditional bioassay with the treatment result.

The results from the single-dose assays were compared with results from traditional six-dose assays. The traditional bioassays, performed in this study, were repeated on different generations of the sea lice strains. This gave to some extent varying EC50-results for the same strain and chemical, possibly due to the heterogeneity in chemical resistance between individuals in a strain of sea lice. This heterogeneity was demonstrated for EMB sensitivity in a study by Ljungfeldt *et al.* (Lina Ljungfeldt, personal communication). The degree of variation in sensitivity within a strain of sea lice cannot be fully characterized as long as resistance mechanisms are unknown. Thirty sea lice chosen for a bioassay will to a varying degree be a representative selection from the population. This will depend on the degree of resistance in the population.

Natural variation has also shown to cause varying LC50-values (the concentration killing 50% of the parasites) in other arthropods (Robertson *et al.* 1995). This can lead to a substantial variation between groups employed in bioassays. It can

also result in a random change in sensitivity between different generations; particularly in a laboratory situation where a limited number of sea lice are chosen to parent the next generation.

A shift towards higher sensitivity can be seen when the resistance factor poses a fitness cost to the parasite. In the absence of a selection pressure, exerted by the chemical, increased sensitivity in subsequent generations may result, as shown for *Lucilia cuprina* (Wiedemann) (McKenzie, Whitten & Adena 1982). Small differences in the way the assays were conducted may also have caused a variation in results. It has been demonstrated that both time of year and gender of the preadult sea lice may possibly influence the results of EMB bioassays (Westcott *et al.* 2008). The EC50-results of this study may possibly have been influenced by one or more of these factors as the bioassays were performed at different times and on different generations of the parasites. Both genders of the parasite were used in the development of the protocol, but only the two preadult life stages. These are also the life stages used in the field test of the optimal separating doses. However, the same protocol may be applied on the adult life stage as well, but possibly giving some differences in the mortality results.

A total shift from sensitive to resistant or vice versa was not evident for any sea lice strain used in this study. For each of the chemicals, the EC50-results, for both the sensitive sea lice strain and the reduced-sensitive strain, always came out in separate clusters. The extent of the difference between the EC50-results for the two strains

varied. This observation is in accordance with results from Chile for *Caligus rogercresseyi*, where EMB resistance persisted in seven generations of sea lice reared in laboratory conditions (Bravo, Sevatdal & Horsberg 2010).

The small-scale treatments gave significant differences ($P < 0.05$) in treatment efficacy between the sensitive and the reduced-sensitive strains of sea lice for all three chemicals. Treatment with deltamethrin and azamethiphos leads to over a 90% reduction in the number of sea lice for LS A. This is the level set by the Norwegian Food Safety Authority for a treatment to be considered successful (Norwegian Food Safety Authority 2011). For both the pyrethroid-insensitive LS R and LS B and the azamethiphos-insensitive LS H, treatment results were below this level. Bath treatments proved appropriate as small-scale treatments. Performing small-scale treatment in glass tanks with deltamethrin may, however, lead to a rapid loss of treatment agent due to attachment of the pyrethroids to the tank walls. Deltamethrin's tendency to attach to glass surfaces was shown in an experiment carried out on 1- to 4-L glass jars used for bioassays (Wheelock *et al.* 2005). This can possibly influence the treatment results with strains being incorrectly classified as resistant.

For EMB, the treatment efficacy, for both the sensitive LS A and the insensitive LS B, was significantly different. However, both were below 90%. The results from the small-scale treatment with EMB may arise from the practical difficulties involved in conducting voluntary in-feed treatments in small groups in the laboratory. This may give suboptimal plasma levels of EMB caused by inadequate feed uptake (Roy *et al.* 2006). Group treatment was, however, chosen in preference to treatment of individual fish because the tanks available for single fish treatment were not optimal for trials of this time length, adaption, treatment and waiting period for results. The low water temperature of 7 °C at the time of the study may also have contributed to a substantial variation in feeding in addition to a delayed onset of the full effect. Another explanation for the suboptimal results may be that the recording of the effect was performed 2 weeks after the treatment was terminated. This timeframe may have been too short, as the maximum efficacy of EMB has been demonstrated 3 weeks after termination of treatment (Gustafson *et al.* 2006). For LS A, all 10 fish in the treatment group were put in

individual tanks after counting and kept for 7 days. During this time, two fish died and two fish were killed at day 5. Of the eight fish where sea lice were counted while the fish were still alive, five sea lice were found distributed on four fish. Thus, a total treatment efficacy of approximately 95% after 3 weeks could be estimated. LS A was evaluated as sensitive based on field treatment results, results from traditional bioassays, the significant difference in the small-scale treatment efficacy and the results from the second counting.

The separation of the sea lice into sensitive strains and strains with reduced sensitivity for each chemical was performed on the basis of results from field treatments, traditional bioassays and small-scale treatments. The results from the 24-h bioassays showed a clear distinction between the sensitive and the reduced-sensitive strains of sea lice for all three chemicals (Figs 2–4). Furthermore, it was possible to determine the optimal separating dose of each delousing agent by calculating the concentration corresponding to the biggest difference between the lower 80% PI for the dose–response curves of the sensitive strain and the corresponding upper 80% PI for the strain with reduced sensitivity. The 80% PI gave the expected mortalities from one single-dose bioassay, with 90% probability that mortality for the sensitive strain would be within or above the 80% PI for this strain, and 90% probability that the mortality for the strain showing reduced sensitivity would be within or below the 80% PI for this strain. These results are valid for the strains of sea lice used in the development of the test but require further validation for other strains of sea lice. Furthermore, corresponding field treatment results are also necessary in order for the method to be regarded as a fully developed single-dose bioassay for field sensitivity testing.

Given that this study describes a protocol for field sensitivity testing, several strains will possibly be tested using the same protocol. These strains will most likely have different degrees of sensitivity within both the sensitive group and the group with reduced sensitivity. This was demonstrated by Westcott *et al.* (2008) as variation between different strains of sea lice employed in EMB bioassays performed in Canada in 2002. The EC₅₀-values varied between 25 and 118 µg L⁻¹ (Westcott *et al.* 2008). These data originate from a period without reports of treatment failures in Canada and are therefore most likely from

sensitive strains of sea lice. Thus, if similar variation is evident amongst the reduced-sensitive strains as well as in the sensitivity towards the other delousing agents, the calculated optimal separating doses might need to be adjusted.

In order for a field bioassay to be considered credible, there must be a correlation between the bioassay results and the efficacy of a treatment with the same agent. A study from Scotland showed a relationship between bioassay results and full-scale treatment (Roth *et al.* 1996). Jones *et al.* (1992) showed a clear association between observed reduced dichlorvos sensitivity in field treatments and results from 24-h bioassays (Jones *et al.* 1992). In a study from Norway, Ireland and Scotland, however, only a partial correlation between results from bioassays on cypermethrin and deltamethrin and episodes of treatment failure were shown (Sevatdal *et al.* 2005).

To fully validate the current protocol for field use, a similar link between bioassays and treatment efficacy must be established. The results from the small-scale treatments clearly pointed towards an obvious correlation between the bioassay and the treatment efficacy. However, due to the fact that the number of strains tested was limited, a larger cohort of field samples is necessary to pin-point the mortality percentages connected to each single-dose that are clinically most relevant for the separation of sea lice strains into sensitive and resistant strains. Field studies are currently in progress. However, the establishment of a link between bioassays and treatment efficacy is not straightforward. Field treatments can be performed using doses and techniques other than those recommended by the manufacturer. Furthermore, treatment failures may be caused by factors other than resistance, such as differences in feed intake between individual fish (Berg & Horsberg 2009). Bioassays may thus fail in predicting the outcome of such treatments.

In the single-dose bioassay described here, sea lice mortality (in %) will be the test outcome, while the most relevant test outcome in traditional bioassays is EC50-values (Sevatdal & Horsberg 2003; Bravo *et al.* 2008; Westcott *et al.* 2008). Because the EC50-value is calculated from a dose–response curve, it is possible to determine a CI for the corresponding EC50-value. However, it is not possible to determine the uncertainty surrounding the single-dose bioassay results unless several bioassay tests are performed at the same

time. The proposed single-dose assay is more vulnerable to outlier results than traditional bioassays. This is due to the fact that only two groups are used to generate the results: the test group and the control group. Evaluation of the single-dose bioassay results may lead to a higher proportion of live parasites being classified as ‘dead’ than in traditional bioassays. The reason for this miscalculation may be that non-attached live parasites can be poured out and thereby incorrectly classified. However, this error was considered as small, as non-attached parasites were rarely seen in the control groups. The outcome of this simple form of classification is that little training is required to perform the test and that the probability of variation in the interpretation of test results is greatly reduced. The test’s simplicity, unambiguous result interpretation and comparison with a control group serve to make it robust.

Single-dose assays have been developed for other parasitic arthropods. Rust *et al.* (2005) determined the dose causing 100% mortality (EC100) in sensitive strains of cat fleas *Ctenocephalides felis* (Bouché) (Rust *et al.* 2005). A strain of cat fleas is, however, likely to be more homogenous than a strain of sea lice. This is due to the fact that each strain of cat fleas in this study was collected as eggs from a single cat. Given that each cat often contains a limited amount of fleas (Slapeta *et al.* 2011), the eggs collected from the fur of a single cat are likely to originate from a single or a few fleas. This is similar to the strains of sea lice cultivated in the laboratory as opposed to those found in the field. In the field, bioassays will be conducted on sea lice with a variety of parents. This will quite possibly give rise to heterogeneity with regard to sensitivity to the chemical treatment and thereby making the concentration giving EC100 inappropriate for single-dose bioassays in sea lice.

Resistance evaluation of sea lice might also be performed as small-scale treatments. An evaluation of hydrogen peroxide resistance was performed in this manner in Scotland in 1999 (Treasurer *et al.* 2000). Small-scale treatments may be challenging particularly under field conditions. This is due to the requirement for special treatment facilities for a smaller group of fish. According to official regulations for sea lice treatments in Norway, treatments will be performed when sea lice levels are low; maximum 0.5 or 1 adult female per fish (Norwegian Food Safety Authority 2009). In

other words, to obtain reliable results, many fish will have to be included in such a small-scale treatment. This form of sensitivity testing will also be more time-consuming and labour-intensive than bioassays. Another complicating factor is that full efficacy may not be clear before one (pyrethroids) to three (EMB) weeks have elapsed. However on the plus side, the results may be more directly transferable to field treatments.

Evaluation of treatment results from a single farm will provide good background information but is essentially unhelpful with respect to sensitivity testing. This is due to the fact that one needs to be aware of the results from the sensitivity testing prior to choosing a treatment. However, these results might be useful for resistance surveillance and epidemiological studies, as well as providing a guideline for neighbouring farms on which type of treatment to use.

As long as the resistance mechanisms are not fully known in sea lice, there is a need for a sensitivity test which is independent of the resistance mechanism (Denholm *et al.* 2002). As part of an integrated pest management programme, bioassays for sensitivity testing of sea lice are useful both for resistance surveillance (Brooks 2009) and as part of the decision-making process prior to treatment. In a suggestion for new Norwegian regulation on sea lice in aquaculture, sensitivity testing is planned to play a greater role than it currently does. Testing prior to treatment and reporting of test results are planned to be made mandatory (Norwegian Food Safety Authority 2012). This study describes the basic steps in the development of a new and simpler bioassay protocol for sensitivity testing in sea lice. The protocol value and the calculated optimal separating doses need to be further assessed in the field to render this bioassay appropriate for field use. Further development of the single-dose bioassay to include all of today's chemical treatment options will also improve the utility of the test.

Acknowledgements

We would like to thank The Research Council of Norway and The Norwegian Seafood Research Fund for founding the project PrevenT (NFR 199778/S40). We would also like to thank Sigmund Sevatdal and Stian Mørch Aaen for providing us with some of the sea lice strains and Josephine Prendergast with help in revising the manuscript.

References

- Berg A.G.T. & Horsberg T.E. (2009) Plasma concentrations of emamectin benzoate after Slice (TM) treatments of Atlantic salmon (*Salmo salar*): differences between fish, cages, sites and seasons. *Aquaculture* **288**, 22–26.
- Berndt O., Meyhofer R. & Poehling H.M. (2004) The edaphic phase in the ontogenesis of *Frankliniella occidentalis* and comparison of *Hypoaspis miles* and *Hypoaspis aculeifer* as predators of soil-dwelling thrips stages. *Biological Control* **30**, 17–24.
- Bjorn P.A. & Finstad B. (1998) The development of salmon lice (*Lepeophtheirus salmonis*) on artificially infected post smolts of sea trout (*Salmo trutta*). *Canadian Journal of Zoology-Revue Canadienne de Zoologie* **76**, 970–977.
- Bowers J.M., Mustafa A., Speare D.J., Conboy G.A., Brimacombe M., Sims D.E. & Burka J.F. (2000) The physiological response of Atlantic salmon, *Salmo salar* L., to a single experimental challenge with sea lice, *Lepeophtheirus salmonis*. *Journal of Fish Diseases* **23**, 165–172.
- Bowers J.M., Speare D.J. & Burka J.F. (2002) The effects of hydrogen peroxide on the stress response of Atlantic salmon (*Salmo salar*). *Journal of Veterinary Pharmacology and Therapeutics* **25**, 311–313.
- Boxshall G.A. & Bravo S. (2000) On the identity of the common *Caligus* (Copepoda: Siphonostomatoida: Caligidae) from salmonid netpen systems in southern Chile. *Contributions to Zoology* **69**, 137–146.
- Bravo S. (2003) Sea lice in Chilean salmon farms. *Bulletin of the European Association of Fish Pathologists* **23**, 197–200.
- Bravo S., Sevatdal S. & Horsberg T.E. (2008) Sensitivity assessment of *Caligus rogercresseyi* to emamectin benzoate in Chile. *Aquaculture* **282**, 7–12.
- Bravo S., Sevatdal S. & Horsberg T.E. (2010) Sensitivity assessment in the progeny of *Caligus rogercresseyi* to emamectin benzoate. *Bulletin of the European Association of Fish Pathologists* **30**, 99–105.
- Brooks K.M. (2009) Considerations in developing an integrated pest management programme for control of sea lice on farmed salmon in Pacific Canada. *Journal of Fish Diseases* **32**, 59–73.
- Burridge L., Weis J.S., Cabello F., Pizarro J. & Bostick K. (2010) Chemical use in salmon aquaculture: a review of current practices and possible environmental effects. *Aquaculture* **306**, 7–23.
- Carvajal J., Gonzalez L. & George-Nascimento M. (1998) Native sea lice (Copepoda: Caligidae) infestation of salmonids reared in netpen systems in southern Chile. *Aquaculture* **166**, 241–246.
- Costello M.J. (2009a) How sea lice from salmon farms may cause wild salmonid declines in Europe and North America and be a threat to fishes elsewhere. *Proceedings of the Royal Society B-Biological Sciences* **276**, 3385–3394.
- Costello M.J. (2009b) The global economic cost of sea lice to the salmonid farming industry. *Journal of Fish Diseases* **32**, 115–118.
- Denholm I., Devine G.J., Horsberg T.E., Sevatdal S., Fallang A., Nolan D.V. & Powell R. (2002) Analysis and management of resistance to chemotherapeutants in salmon

- lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Pest Management Science* **58**, 528–536.
- Finstad B., Bjorn P.A., Grimnes A. & Hvidsten N.A. (2000) Laboratory and field investigations of salmon lice [*Lepeophtheirus salmonis* (Kroyer)] infestation on Atlantic salmon (*Salmo salar* L.) post-smolts. *Aquaculture Research* **31**, 795–803.
- Grant A.N. (2002) Medicines for sea lice. *Pest Management Science* **58**, 521–527.
- Grave K., Horsberg T.E., Lunestad B.T. & Litleskare I. (2004) Consumption of drugs for sea lice infestations in Norwegian fish farms: methods for assessment of treatment patterns and treatment rate. *Diseases of Aquatic Organisms* **60**, 123–131.
- Grimnes A. & Jakobsen P.J. (1996) The physiological effects of salmon lice infection on post-smolt of Atlantic salmon. *Journal of Fish Biology* **48**, 1179–1194.
- Gustafson L., Ellis S., Robinson T., Marengi F. & Endris R. (2006) Efficacy of emamectin benzoate against sea lice infestations of Atlantic salmon, *Salmo salar* L.: evaluation in the absence of an untreated contemporary control. *Journal of Fish Diseases* **29**, 621–627.
- Johnson S.C. & Albright L.J. (1991) Development, growth, and survival of *Lepeophtheirus salmonis* (Copepoda, Caligidae) under laboratory conditions. *Journal of the Marine Biological Association of the United Kingdom* **71**, 425–436.
- Jones M.W., Sommerville C. & Wootten R. (1992) Reduced sensitivity of the salmon louse, *Lepeophtheirus salmonis*, to the organophosphate dichlorvos. *Journal of Fish Diseases* **15**, 197–202.
- Lees F., Baillie M., Gettinby G. & Revie C.W. (2008) The efficacy of emamectin benzoate against infestations of *Lepeophtheirus salmonis* on farmed Atlantic salmon (*Salmo salar* L) in Scotland, 2002–2006. *PLoS One* **3**, e1549.
- Mckenzie J.A., Whitten M.J. & Adena M.A. (1982) The Effect of genetic background on the fitness of diazinon resistance genotypes of the Australian sheep blowfly, *Lucilia-Cuprina*. *Heredity* **49**, 1–9.
- Midtlyng P.J., Grave K. & Horsberg T.E. (2011) What has been done to minimize the use of antibacterial and antiparasitic drugs in Norwegian aquaculture? *Aquaculture Research* **42**, 28–34.
- Murray A.G. (2011) A simple model to assess selection for treatment-resistant sea lice. *Ecological Modelling* **222**, 1854–1862.
- Norwegian Food Safety Authority (2009) Sea lice regulations for aquaculture (Forskrift om bekjempelse av lus i akvakulturanlegg) Paragraph §5. Available at http://lovdata.no/cgi-wift/wifldles?doc=/app/gratis/www/docroot/for/sf/fi/fi-20090818-1095.html&emne=luseforskrift* (Accessed June 2012).
- Norwegian Food Safety Authority (2011) Regulations for additional requirements for the treatment against sea lice as determined for the first half of 2012 (Forskrift om ytterligere krav til behandling mot lakselus første halvår 2012) Paragraph §3. Available at http://lovdata.no/cgi-wift/wifldles?doc=/app/gratis/www/docroot/for/sf/fi/fi-20111219-1401.html&emne=lakselus* (Accessed June 2012).
- Norwegian Food Safety Authority (2012) Draft for regulations for suppression of sea lice in aquaculture (Utkast til forskrift om bekjempelse av lakselus i akvakulturanlegg) Paragraph §9 and §10. Available at http://www.mattilsynet.no/mattilsynet/multimedia/archive/00078/Forslag_til_ny_lakse_78759a.pdf (Accessed June 2012).
- Pike A.W. & Wadsworth S.L. (2000) Sealice on salmonids: their biology and control. *Advances in Parasitology*, **44**, 233–337.
- Robertson J.L., Preisler H.K., Ng S.S., Hickie L.A. & Gelernter W.D. (1995) Natural variation – a complicating factor in bioassays with chemical and microbial pesticides. *Journal of Economic Entomology* **88**, 1–10.
- Roth M., Richards R.H., Dobson D.P. & Rae G.H. (1996) Field trials on the efficacy of the organophosphorus compound azamethiphos for the control of sea lice (Copepoda: Caligidae) infestations of farmed Atlantic salmon (*Salmo salar*). *Aquaculture* **140**, 217–239.
- Roy W.J., Gillan N., Crouch L., Parker R., Rodger H. & Endris R. (2006) Depletion of emamectin residues following oral administration to rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **259**, 6–16.
- Rust M.K., Denholm I., Dryden M.W., Payne P., Blagburn B.L., Jacobs D.E., Mencke N., Schroeder I., Vaughn M., Mehlhorn H., Hinkle N.C. & Williamson M. (2005) Determining a diagnostic dose for imidacloprid susceptibility testing of field-collected isolates of cat fleas (Siphonaptera: Pulicidae). *Journal of Medical Entomology* **42**, 631–636.
- Sevatdal S. & Horsberg T.E. (2003) Determination of reduced sensitivity in sea lice (*Lepeophtheirus salmonis* Kroyer) against the pyrethroid deltamethrin using bioassays and probit modelling. *Aquaculture* **218**, 21–31.
- Sevatdal S., Copley L., Wallace C., Jackson D. & Horsberg T.E. (2005) Monitoring of the sensitivity of sea lice (*Lepeophtheirus salmonis*) to pyrethroids in Norway, Ireland and Scotland using bioassays and probit modelling. *Aquaculture* **244**, 19–27.
- Slapeta J., King J., McDonnell D., Malik R., Homer D., Hannan P. & Emery D. (2011) The cat flea (*Ctenocephalides f. felis*) is the dominant flea on domestic dogs and cats in Australian veterinary practices. *Veterinary Parasitology* **180**, 383–388.
- Treasurer J.W., Wadsworth S. & Grant A. (2000) Resistance of sea lice, *Lepeophtheirus salmonis* (Kroyer), to hydrogen peroxide on farmed Atlantic salmon, *Salmo salar* L. *Aquaculture Research* **31**, 855–860.
- Westcott J.D., Stryhn H., Burka J.F. & Hammell K.L. (2008) Optimization and field use of a bioassay to monitor sea lice *Lepeophtheirus salmonis* sensitivity to emamectin benzoate. *Diseases of Aquatic Organisms* **79**, 119–131.
- Wheelock C.E., Miller J.L., Miller M.J., Phillips B.M., Gee S.J., Tjeerdema R.S. & Hammock B.D. (2005) Influence of container adsorption upon observed pyrethroid toxicity to *Ceriodaphnia dubia* and *Hyaella azteca*. *Aquatic Toxicology* **74**, 47–52.

Received: 29 June 2012

Revision received: 10 October 2012

Accepted: 17 October 2012

II



Contents lists available at [ScienceDirect](http://www.sciencedirect.com)

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep



Short communication

First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway

Kari Olli Helgesen^{a,*}, Harriet Romstad^b, Stian Mørch Aaen^a, Tor Einar Horsberg^a

^a Norwegian University of Life Sciences, School of Veterinary Science, Sea Lice Research Centre, Oslo, Norway

^b Aqua Kompetanse AS, Flatanger, Norway

ARTICLE INFO

Article history:

Received 27 October 2014
Received in revised form
30 December 2014
Accepted 11 January 2015
Available online xxx

Keywords:

Salmon lice
Lepeophtheirus salmonis
Sensitivity
Hydrogen peroxide
Bioassay

ABSTRACT

Reduced sensitivity towards chemotherapeutants in the salmon louse *Lepeophtheirus salmonis* (Krøyer) is an increasing problem for the fish farming industry. Most fish farmers are dependent on chemical treatments in order to maintain salmon lice numbers below permitted levels. However parasites showing reduced sensitivity contribute to complicating this task. Hydrogen peroxide (H₂O₂) is used as a delousing agent in bath treatments and until recently treatment failures due to reduced H₂O₂-sensitivity have not been documented in Norway. The aim of the current study was to develop a bioassay protocol suitable for testing H₂O₂-sensitivity in *L. salmonis*. If failed treatments were found to be caused by parasite insensitivity to H₂O₂ the possibility of this reduced sensitivity being hereditary was looked into. The results show that bioassays permit differentiation between strains of salmon lice with regards to H₂O₂-sensitivity, coinciding with treatment efficacies. Up to ten times variance in sensitivity between two strains was recorded. The progeny of the least sensitive salmon lice also showed reduced sensitivity to H₂O₂ in a bioassay, which indicates that reduced sensitivity towards H₂O₂ is hereditary. The current study presents the first case report of reduced sensitivity towards H₂O₂ in salmon lice in Norway. This change in sensitivity imposes a threat to the Norwegian fish farming industry and should be monitored closely.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The salmon (or sea) louse *Lepeophtheirus salmonis* (Krøyer) infests both wild and farmed salmonids in the Northern Hemisphere (Costello, 2006; Pike and Wadsworth, 2000; Torrissen et al., 2013). Control of the salmon lice in fish farms is important in order to protect the farmed fish from parasite-related stress and injuries as well as minimizing the infestation pressure on wild salmonids. Most fish farms are dependent on chemical treatments in order to keep the parasite numbers below national maximum permitted levels, but few chemical treatment agents are available (Burka et al., 1997; Roth, 2000; Westcott et al., 2004). This has subsequently led to the development of reduced sensitivity in *L. salmonis* towards most of the available chemical treatments (Jones et al., 1992; Lees et al., 2008a; Roth et al., 1996; Sevattal and Horsberg, 2003; Treasurer et al., 2000). The geographical dispersion of the reduced sensitivity towards each treatment agent

varies (Grøntvedt et al., 2014; Jones et al., 2013; Lees et al., 2008b). Chile, the major salmonid producing country in the Southern Hemisphere is having problems with the sea louse *Caligus rogercresseyi* (Johnson et al., 2004). In Chile, chemical treatments are essential in order to control sea lice levels; however this task has become increasingly difficult as the parasites have developed reduced sensitivity towards both pyrethroids and the avermectin emamectin benzoate (Bravo et al., 2008, 2013; Helgesen et al., 2014).

In Norway, hydrogen peroxide (H₂O₂) was used to a certain extent as a delousing agent between the years 1993 and 1997 (Grave et al., 2004). This use was terminated due to the introduction of more efficient chemicals with larger safety margins. The emerging occurrence of reduced sensitivity towards other compounds however led to the re-introduction of H₂O₂ for salmon lice treatment in 2009 (Norwegian Institute of Public Health, 2014). In order to delay the development of reduced sensitivity, an increased range of chemical treatment agents is desirable. This permits rotation between compounds with various modes of action subsequently postponing the development of reduced sensitivity (Brooks, 2009; Denholm et al., 2002). Unfortunately, the extensive use of H₂O₂ as a delousing agent in various parts of Norway (Grøntvedt et al., 2014) has increased the risk of developing reduced sensitivity towards

* Corresponding author at: Norwegian University of Life Sciences, School of Veterinary Science, Pb 8146 Dep, 0033 Oslo, Norway. Tel.: +47 22597090.
E-mail address: kari.helgesen@nmbu.no (K.O. Helgesen).

<http://dx.doi.org/10.1016/j.aqrep.2015.01.001>

2352-5134/© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

this compound. In Scotland, reduced sensitivity towards H_2O_2 in salmon lice was reported in the year 2000, following extensive use of this treatment agent over several years (Treasurer et al., 2000). Due to the toxicity and low safety margin of H_2O_2 in fish, only minor increases in treatment time and concentration can be introduced (Bruno and Raynard, 1994; Thomassen, 1993). In order to avoid unsuccessful treatments due to reduced parasitic sensitivity, a method for sensitivity testing in salmon lice is therefore required.

Incidences of reduced treatment efficacy following H_2O_2 -treatments in Norway have raised the issue of possible reduced sensitivity. Treatment failure may be due to inadequate delousing procedures or reduced sensitivity in the parasites (Denholm et al., 2002). Biological assays (bioassays) are employed to test for reduced sensitivity in arthropods when the resistance mechanisms are unknown (Robertson et al., 2007). Bioassays have been developed for sensitivity testing in *L. salmonis* towards pyrethroids, emamectin benzoate and the organophosphate azamethiphos (Helgesen and Horsberg, 2013; Sevatdal and Horsberg, 2003; Westcott et al., 2008; Whyte et al., 2013). Treasurer et al. (2000) tested for H_2O_2 -sensitivity using small scale treatments, however this method requires the use of fish and due to animal welfare and practical reasons should be avoided if possible.

Possible mechanisms for reduced H_2O_2 -sensitivity in salmon lice include increased antioxidant enzymes activity such as catalase, glutathione peroxidase or glutathione-S-transferase, all of which have been found in mammalian cells in conjunction with H_2O_2 -resistance (Fiander and Schneider, 2000; Spitz et al., 1992; Baud et al., 2004). Increased catalase activity has also been seen in H_2O_2 -resistant bacteria and fungi (Amin and Olson, 1968; Elkins et al., 1999; Nakamura et al., 2012; Uhlich, 2009). Other possible enzymes involved in reduced H_2O_2 sensitivity are superoxide dismutase, superoxide reductase, glutathione reductase and thioredoxin, as they are proven to possess activity against reactive oxygen species (Nordberg and Arner, 2001).

The primary objective of the present study was to develop bioassay protocols suitable for H_2O_2 -bioassays. Whether reduced treatment effects from H_2O_2 -treatments were caused by reduced salmon lice sensitivity was also investigated. This required using bioassays on both field collected parasites and their laboratory reared progeny.

2. Materials and methods

2.1. Salmon lice

Salmon lice originating from seven different farms in Norway were employed in the current study (Table 1). All salmon lice with the exception of Ls A originated from the northern part of Mid-Norway. Ls V came from a farm reporting of reduced treatment efficacy in H_2O_2 -treatment since one year back. The treatment performed three months prior to parasite collection for field bioassays had 74.1% efficacy in mobile stages (salmon lice from 80 fish collected from 4 fish cages were counted on the day of treatment and on the following day to calculate efficacy). The treatment efficacy was 51.4% in mobile stages in the treatment performed one month before the Ls V F0 bioassay was performed (salmon lice from 30 fish from 3 cages were counted on two consecutive days, with treatment on day 1, to evaluate treatment efficacy). Ls Ky, Ls D and Ls Aa all came from farms with a history of H_2O_2 -treatments against salmon lice, but without having experienced reduced treatment efficacies. Ls S and Ls Kl originated from the same area, but from farms which had not treated with H_2O_2 themselves. Ls A was a laboratory strain originally collected from the northern part of North-Norway in 2011. Bioassays and small scale treatments had

shown this strain to be sensitive to pyrethroids, azamethiphos and emamectin benzoate (Helgesen and Horsberg, 2013). Ls A had never been exposed to H_2O_2 , neither in field nor in the laboratory.

The salmon lice designated for field bioassays were collected from anesthetized fish at the sea farms and transported in cooled sea water to the laboratory. These bioassays, six in total, were performed by Aqua Kompetanse AS at their laboratory in Flatanger, Norway. The other five bioassays were performed at The Norwegian University of Life Sciences in Oslo, Norway. Table 1 provides the details. The salmon lice for the five latter assays were reared on fish at the NIVA Marine Research Station in Drøbak, Norway. Ls A was kept in a continuous culture on Atlantic salmon (*Salmo salar*), while Ls V was sent to the laboratory as egg strings. After hatching and development into copepodites, 20 sea trout weighing about 150 g each were infested with the parasites. Approximately 50 copepodites per fish were employed for the infestation, which was conducted in 30 l of aerated sea water for 45 min. Pre-adult parasites and adult males were used for the bioassays depending on which instars were available at the time. All bioassays were initiated within 8 h after parasite collection.

2.2. Bioassays

Two types of bioassays were performed: 30-min and 24-h bioassays. The 30-min bioassays were performed according to the protocol for pyrethroid bioassays described in Sevatdal and Horsberg (2003) with some modifications. The parasites were exposed to between six and twelve different concentrations of H_2O_2 using Interlox Paramove 50 (H_2O_2 50%, w/w, Solvay Chemicals, Belgium) in Oslo and Eka HP T49 S (H_2O_2 49.7%, w/w, Azko Nobel, Sweden) in Flatanger, diluted in sea water. Nominal concentrations ranging from 0 to 5000 mg L⁻¹ were utilized and varying concentrations within this range were chosen according to the expected sensitivity level of the respective salmon lice strain. The salmon lice were distributed in sea water filled polystyrene bioassay boxes, with approximately 10 parasites (6–13) in each box. The different concentrations of H_2O_2 were prepared by adding the appropriate amount of H_2O_2 to cooled sea water (10–12 °C) in one litre polypropylene containers. In each of the concentrations applied in each bioassay, two of the bioassay boxes were submerged. For the field bioassays with Ls Ky, Ls D, Ls Aa and Ls Kl only one box of parasites was used for the control group. The boxes were kept in the solution for 30 min and the results were immediately recorded. Parasites attached to the wall of the box or swimming in a straight line were considered alive. All others were regarded as immobilized or dead. For the bioassays on Ls A and Ls V F1 the boxes containing the parasites were relocated to fresh sea water after the initial evaluation, without removing any parasites, and kept at 12 °C under constant aeration for the following 24 h. The results were then re-evaluated.

The 24-h bioassays were performed according to the protocol for pyrethroids, azamethiphos and emamectin benzoate bioassays described in Helgesen and Horsberg (2013), with some modifications. Between eight and seventeen parasites from Ls A were exposed to six or seven different nominal concentrations of H_2O_2 , ranging between 0 and 120 mg L⁻¹, in one litre glass bottles. The bottles were kept at 12 °C for 24 h and supplied with constant aeration. Ls V was exposed to 11 different nominal H_2O_2 -concentrations between 0 and 1800 mg L⁻¹. Between 30 and 61 parasites were used for each concentration. After the exposure period the results were recorded by turning the bottles upside down three times and then moving them in circles with a diameter of 20 cm 10 times. When the water had settled it was poured out into a beaker. All parasites remaining in the bottle or able to attach to the beaker wall or swim in a straight line were considered alive. All other parasites were regarded as immobilized or dead.

Table 1

Bioassays. All salmon lice strains were collected in the northern part of Mid-Norway with the exception of Ls A which came from the northern part of North-Norway. Ls V F0 and Ls V F1 were the same strain, but bioassays were performed both with parasites from the field (F0) and with laboratory-reared parasites from field collected egg strings (F1). Two types of bioassays were performed, with both 30 min and 24 h parasite exposure to hydrogen peroxide. Six to eleven different concentrations were employed for each bioassay and the dose-response curve were modelled in JMP (SAS Institute Inc., Cary, NC, USA). The concentrations in mg L⁻¹ immobilizing 50% of the parasites (EC₅₀) with 95% confidence intervals (CI) are given.

Strain	Origin	Date	Place	Assay	EC ₅₀	Lower 95% CI	Upper 95% CI
Ls A	Laboratory	10.10.2013	Oslo	30-min	216	153	305
Ls V F1	Laboratory	13.11.2013	Oslo	30-min	1767	1494	2090
Ls V F0	Field	11.10.2013	Flatanger	30-min	2127	1253	3610
Ls S	Field	03.02.2014	Flatanger	30-min	539	386	754
Ls Ky	Field	03.02.2014	Flatanger	30-min	693	483	993
Ls D	Field	31.01.2014	Flatanger	30-min	563	435	730
Ls Aa	Field	30.01.2014	Flatanger	30-min	541	403	727
Ls Kl	Field	30.01.2014	Flatanger	30-min	538	373	776
Ls A	Laboratory	12.02.2014	Oslo	24-h	45.9	29.8	70.9
Ls A	Laboratory	23.06.2014	Oslo	24-h	64.7	43.4	96.4
Ls V F1	Laboratory	13.11.2013	Oslo	24-h	138	112	171

The bioassay results were modelled using probit-analysis in JMP 10.0.0 (SAS Institute Inc., Cary, NC, USA) to find EC₅₀-values, which is the concentration immobilizing 50% of the parasites, with 95% confidence intervals (CI).

3. Results

Ls A had the lowest EC₅₀-values for both the 30-min and the 24-h bioassays with 216 and 45.9 to 64.7 mg L⁻¹ respectively. Ls V had the highest EC₅₀-values for both types of bioassays, with EC₅₀-values of 1767 and 2127 mg L⁻¹ in the 30-min bioassays and 138 mg L⁻¹ in the 24-h bioassay.

The five other strains tested in the 30-min bioassay showed intermediate EC₅₀-values, ranging from 538 to 693 mg L⁻¹. The dose-response curves for Ls A and Ls V F1 with 90% CI are presented in Fig. 1 (30 min exposure) and Fig. 2 (24 h exposure). All results after modelling are displayed in Table 1.

No control group mortality was seen in any of the bioassays with 30 min exposure and immediate evaluation. In the 24-h bioassay, 0 and 8.3% mortality was observed in the Ls A control groups, while 7.5% of the parasites in the Ls V-bioassay-control group were dead after 24 h.

With respect to the results for the 30-min Ls A and Ls V F1 bioassays, fewer parasites were regarded as dead or immobilized at the re-evaluation 24 h after exposure than in the first evaluation. With respect to Ls A the total percentage immobilized parasites decreased from 68 to 51% when the results from all concentrations were included. For Ls V F1 the same group decreased from 48 to 21%. The attempts to model the re-evaluation results gave illogical dose-response curves.

4. Discussion

Assuming that H₂O₂-sensitivity is similar in both attached and free-swimming parasites; one would expect reduced treatment efficacy from treatment of fish infested with Ls V. The treatment regime, outlined in the summary of product characteristics for Paramove (49.5% H₂O₂, Solvay chemicals), is exposure to 1500 mg L⁻¹ H₂O₂ for 20 min (Norwegian Medicines Agency, 2014). In the Ls V bioassays, less than 50% of the parasites were immobilized when exposed to this concentration for 30 min. The results from the bioassays may not however be directly interpreted into treatment results. The bioassays were performed on parasites detached from the fish and sensitivity to chemicals may differ between parasites attached to and detached from the fish (Sevatdal and Horsberg, 2003). Furthermore during treatment, a constant H₂O₂-level is maintained through-out the exposure time. In contrast the bioassays were performed by adding the substance

to sea water only at the beginning of exposure. In order to develop bioassays into an accurate prediction tool for treatment efficacy, the correlation between bioassay results and treatment results need to be elucidated.

The intermediate bioassay results found in five of the tested strains could be a sign of reduced sensitivity. This hypothesis is supported by the fact that all of the lower 95% CI values in these strains were above the upper 95% CI for the fully sensitive strain, Ls A. The intermediate results could also represent the range of EC₅₀-results found in sensitive parasites. This latter theory is supported by the results obtained by Bruno and Raynard (1994). Evaluating their bioassay results using probit analysis in JMP, an EC₅₀-value of 890 mg L⁻¹ could be calculated for pre-adult parasites and 503 mg L⁻¹ for adult salmon lice. In an experiment where salmon lice infested salmon were treated with different concentrations of H₂O₂, an EC₅₀-value of 800 mg L⁻¹ was obtained (Thomassen, 1993). In both trials results were evaluated immediately after exposure. However the shorter exposure period in these trials (20 min) would have increased the EC₅₀-values. This in comparison to a protocol with 30 min exposure time. Furthermore both experiments were performed at lower temperatures (10 °C in Bruno and Raynard's experiment and 6–9 °C in Thomassen's experiment) than in the current study, which might also have affected the results. The toxic effect of H₂O₂ on salmon lice increases at higher temperatures (Treasurer and Grant, 1997). EC₅₀-values would therefore be expected to be higher at lower temperatures. Thomassen's experiment was performed on parasites attached to fish. Given that salmon lice are more susceptible to pyrethroid bath treatments when detached from fish, (Sevatdal and Horsberg, 2003) and if this is also the case for H₂O₂, then the same parasites would have shown a lower EC₅₀-value if Thomassen's experiment had been performed on parasites which had been removed from the fish. Whether the differences in EC₅₀-values between different life stages of salmon lice, as seen by Bruno and Raynard (1994), were present in the current study, was not evaluated. If differences were present this might have biased the results as the parasitic instars were not allocated evenly within or between the experiments. Future research should look into the possible differences of H₂O₂-sensitivity between instars of *L. salmonis* as this might influence both sensitivity assessments and treatment effects.

A large proportion of the parasites immediately characterized as immobilized, were 24 h later re-evaluated as alive. Recovering parasites after H₂O₂-exposure were also observed by Hodneland et al. (1993), Johnson et al. (1993) and Treasurer and Grant (1997). This implies that salmon lice have mechanisms to fight the effect of H₂O₂. These mechanisms seem to be induced by the treatment, as sensitive parasites are immediately knocked down and

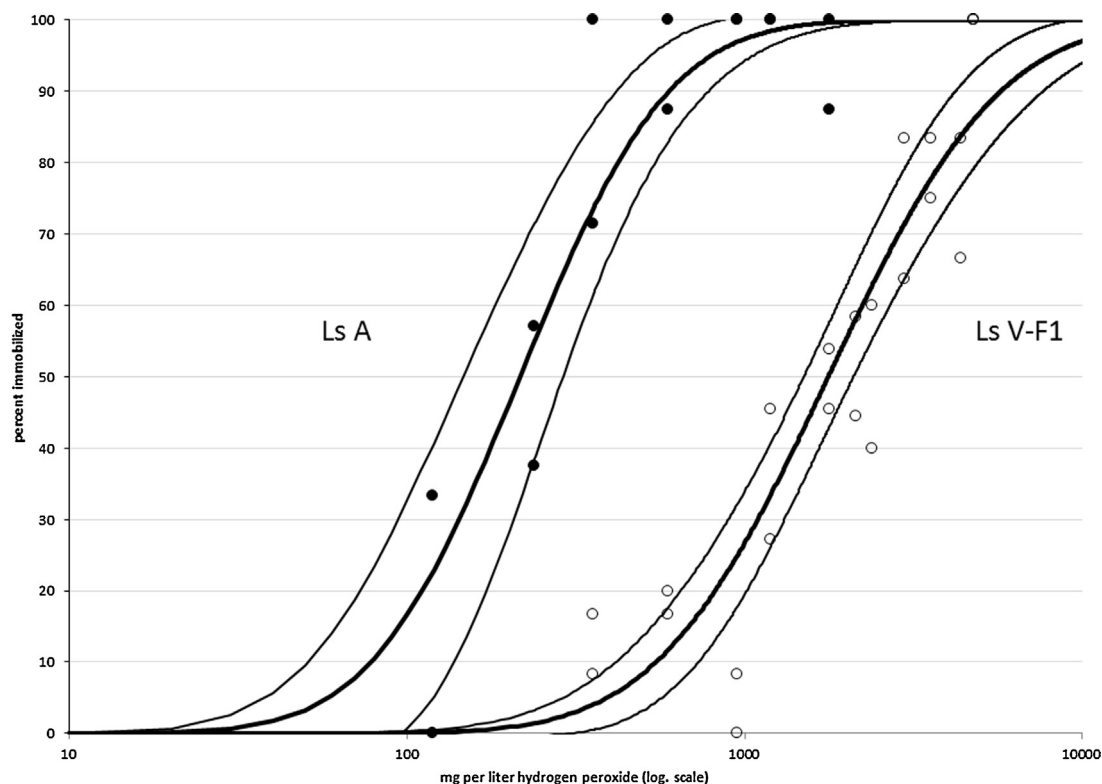


Fig. 1. 30-min bioassay. The dose-response curves (90% CI) and the observed immobilization rates for two of the salmon lice strains tested for sensitivity towards hydrogen peroxide in a 30-min bioassay. The observed rates for the sensitive strain (Ls A) are indicated by filled circles, while the corresponding values for the strain with reduced sensitivity (Ls V) are indicated by open circles.

later recover. Recovery, following treatment-induced immobilization, was observed to a greater extent in parasites from the least sensitive strain. This was in addition to the fact that a higher concentration of H_2O_2 was necessary for their immobilization. The cause of this might be dual; both an increase in the already existing inducible mechanism and an additional mechanism that is present prior to exposure. The exact mechanisms behind reduced sensitivity towards H_2O_2 in salmon lice are not yet clarified, and research into this field is therefore required. The knowledge on mechanisms for reduced sensitivity is important in order to develop mechanism-specific tools for sensitivity testing and -surveillance, as well as avoiding pursuing existing mechanisms when developing new chemical treatment agents (Denholm et al., 2002).

Due to the revitalization of the parasites the results from the 30 min-exposure bioassays could not be evaluated in the same manner as the Sevattal and Horsberg (2003) pyrethroid bioassays. Sevattal and Horsberg allowed a waiting period of 24 h in fresh sea water following the exposure period. When this protocol was applied to the H_2O_2 -bioassays, it resulted in illogical dose-response curves. The immediate evaluation of the bioassays however gave plausible dose-response curves in addition to low control group mortalities. This was therefore chosen for the H_2O_2 -bioassay protocol. The chosen protocol was considered a good proxy for lice survival under farm treatment conditions due to similar exposure periods. The parasites that recovered after first being immobilized were not considered important with regards to field treatments, as they were not seen to re-infest fish in the study by Treasurer and Grant. This coincides with the Norwegian field treatment experience. However, a laboratory study showed that both adult males and pre-adult parasites have the ability to re-infest salmon independent of the parasites' previous exposure to H_2O_2 (McAndrew et al., 1998).

The 24-h bioassay also differentiated between parasite strains with differences in sensitivity. The EC_{50} -value for the least sensitive strain was two to three times higher than for the most sensitive strain. The lower difference in EC_{50} -value, compared to the 30-min bioassays, might be caused by the mechanisms for reduced sensitivity and/or H_2O_2 -degradation. Since exposure time does not exceed 30 min in normal H_2O_2 field treatments, it may be concluded that the parasite has not undergone selection in order to develop mechanisms to withstand long term H_2O_2 -exposure. Developing a 24-h bioassay is an attempt to standardize all salmon lice bioassays to the set up by Helgesen and Horsberg (2013), thereby making field bioassays easier to perform. To accomplish this task a degradation curve of H_2O_2 under the circumstances given in the bioassay protocol should be employed and the correlation between bioassays and treatment results should be established.

The development of reduced sensitivity towards H_2O_2 in *L. salmonis* was expected, as reduced sensitivity towards pyrethroids, azamethiphos and emamectin benzoate has previously been described by bioassays performed in Norway (Grøntvedt et al., 2014; Helgesen and Horsberg, 2013; Sevattal et al., 2005; Sevattal and Horsberg, 2003). As a result of the development of reduced sensitivity towards other available chemical treatments, the use of H_2O_2 for anti-salmon lice treatments has increased since the product was re-introduced to the Norwegian market in 2009. In 2013, 8262 metric tonnes of H_2O_2 was applied in treatment while the figure was 2538 metric tonnes the previous year (Norwegian Institute of Public Health, 2014). The outbreak of amoebic gill disease (AGD) may also have contributed to the increased use of H_2O_2 in 2013. Treatments against AGD will nonetheless simultaneously combat infested salmon lice and therefore impose selection pressure on both parasitic species. Grøntvedt et al. (2014) showed that the most intense H_2O_2 treatment regime against salmon lice was found in the northern part of Mid-Norway and in the southern part of

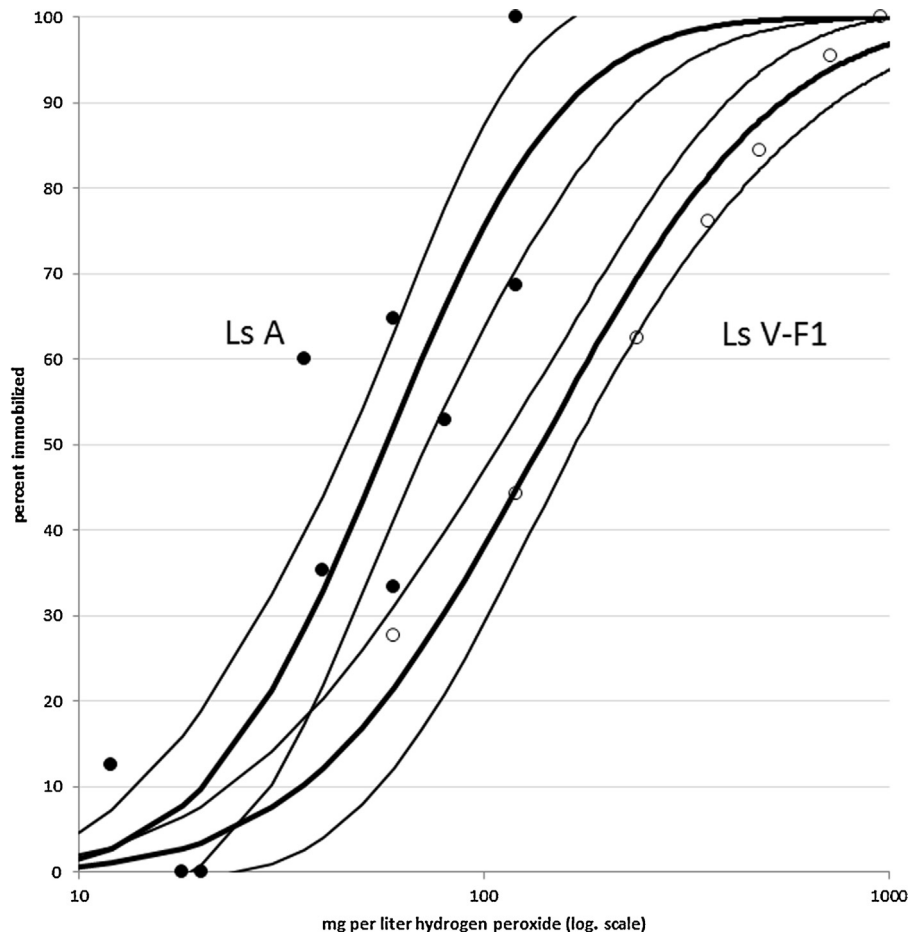


Fig. 2. 24-h bioassay. The dose-response curves (90% CI) and the observed immobilization rates for the two strains of salmon lice tested for sensitivity towards hydrogen peroxide in a 24-h bioassay. The observed rates for the sensitive strain (Ls A) are indicated by filled circles, while the corresponding values for the strain with reduced sensitivity (Ls V) are indicated by open circles. The dose-response curve for Ls A is modelled with results from two different bioassays.

North-Norway. The development of reduced sensitivity towards H_2O_2 in these particular areas was therefore not unexpected as repeated treatments provide fast lane evolution towards reduced sensitivity. In the current study reduced H_2O_2 -sensitivity was found in one strain of salmon lice in combination with reduced treatment efficacy. To decide if reduced sensitivity is a settled trait in the salmon lice population more sensitivity tests should be conducted on farms reporting of reduced treatment efficacy.

Reduced H_2O_2 -sensitivity in *L. salmonis*, due to extensive use of the chemical as a delousing agent, was first reported from Scotland in 1999, seven years after H_2O_2 -treatment was introduced to the Scottish market (Treasurer et al., 2000). Using experimental bin treatments, 15% effect of treatment was recorded for the gravid females and 25% effect for the other mobile stages. Fish, infested with salmon lice previously unexposed to H_2O_2 , were treated and used as a control with an effect of 87% or more for all groups. The study by Treasurer et al. was performed on the F0-generation of salmon lice, while the current study also included the F1-generation, which had not previously been exposed to H_2O_2 . This structure provided stronger evidence for the heritability of reduced H_2O_2 -sensitivity.

The bioassay results presented in the current paper show differences in the sensitivity level towards H_2O_2 between various strains of *L. salmonis*. These differences coincide with the differences in treatment efficacies. 30-min bioassays revealed up to tenfold variations in EC_{50} -values between the different strains of salmon lice. Reduced sensitivity towards H_2O_2 , evident in both reduced treatment efficacy and increased bioassay EC_{50} -values, is

most likely an inherited trait. This is suggested by the fact that the F1-generation showed a relatively high EC_{50} -value, despite having not been exposed to H_2O_2 -treatment. The current study presents the first case report of reduced H_2O_2 -sensitivity in salmon lice in Norway. This reduced sensitivity imposes a threat to the Norwegian fish farming industry and should be monitored closely.

Acknowledgement

The current study was financed by the SFI-Sea Lice Research Centre (NFR 203513/030) platform, the project Prevent (NFR 199778/S40), financed by The Research Council of Norway and The Norwegian Seafood Research Fund, and by Aqua kompetanse AS. We would also like to thank the fish farming companies that facilitated the collection of parasites and Josephine Prendergast for proof-reading the manuscript.

References

- Amin, V.M., Olson, N.F., 1968. Influence of catalase activity on resistance of coagulase-positive staphylococci to hydrogen peroxide. *Appl. Microbiol.* 16, 267–270.
- Baud, O., Greene, A.E., Li, J.R., Wang, H., Volpe, J.J., Rosenberg, P.A., 2004. Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *J. Neurosci.* 24, 1531–1540.
- Bravo, S., Nuñez, M., Silva, M.T., 2013. Efficacy of the treatments used for the control of *Caligus rogercresseyi* infecting Atlantic salmon, *Salmo salar* L., in a new fish-farming location in Region XI, Chile. *J. Fish Dis.* 36, 221–228.
- Bravo, S., Sevattal, S., Horsberg, T.E., 2008. Sensitivity assessment of *Caligus rogercresseyi* to emamectin benzoate in Chile. *Aquaculture* 282, 7–12.

- Brooks, K.M., 2009. Considerations in developing an integrated pest management programme for control of sea lice on farmed salmon in Pacific Canada. *J. Fish Dis.* 32, 59–73.
- Bruno, D.W., Raynard, R.S., 1994. Studies on the use of hydrogen peroxide as a method for the control of sea lice on Atlantic salmon. *Aquac. Int.* 2, 10–18.
- Burka, J.F., Hammell, K.L., Horsberg, T.E., Johnson, G.R., Rainnie, D.J., Speare, D.J., 1997. Drugs in salmonid aquaculture – a review. *J. Vet. Pharmacol. Ther.* 20, 333–349.
- Costello, M.J., 2006. Ecology of sea lice parasitic on farmed and wild fish. *Trends Parasitol.* 22, 475–483.
- Denholm, I., Devine, G.J., Horsberg, T.E., Sevatdal, S., Fallang, A., Nolan, D.V., Powell, R., 2002. Analysis and management of resistance to chemotherapeutants in salmon lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Pest Manage. Sci.* 58, 528–536.
- Elkins, J.G., Hassett, D.J., Stewart, P.S., Schweizer, H.P., McDermott, T.R., 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Appl. Environ. Microbiol.* 65, 4594–4600.
- Fiander, H., Schneider, H., 2000. Dietary ortho phenols that induce glutathione S-transferase and increase the resistance of cells to hydrogen peroxide are potential cancer chemopreventives that act by two mechanisms: the alleviation of oxidative stress and the detoxification of mutagenic xenobiotics. *Cancer Lett.* 156, 117–124.
- Grave, K., Horsberg, T.E., Lunestad, B.T., Litleskare, I., 2004. Consumption of drugs for sea lice infestations in Norwegian fish farms: methods for assessment of treatment patterns and treatment rate. *Dis. Aquat. Organ.* 60, 123–131.
- Grøntvedt, R.N., Jansen, P., Horsberg, T.E., Helgesen, K.O., Tarpai, A., 2014. The Surveillance Programme for Resistance to Chemotherapeutants in Salmon Lice (*Lepeophtheirus salmonis*) in Norway 2013. Norwegian Veterinary Institute, Oslo.
- Helgesen, K.O., Bravo, S., Sevatdal, S., Mendoza, J., Horsberg, T.E., 2014. Deltamethrin resistance in the sea louse *Caligus rogercresseyi* (Boxhall and Bravo) in Chile: bioassay results and usage data for antiparasitic agents with references to Norwegian conditions. *J. Fish Dis.* 37, 877–890.
- Helgesen, K.O., Horsberg, T.E., 2013. Single-dose field bioassay for sensitivity testing in sea lice, *Lepeophtheirus salmonis*: development of a rapid diagnostic tool. *J. Fish Dis.* 36, 261–272.
- Hodneland, K., Nylund, A., Nilsen, F., Midttun, B., 1993. The effect of Nuvan, azame-thiphos and hydrogen peroxide on salmon lice (*Lepeophtheirus salmonis*). *Bull. Eur. Assoc. Fish Pathol.* 13, 203–206.
- Johnson, S.C., Constible, J.M., Richard, J., 1993. Laboratory investigations on the efficacy of hydrogen peroxide against the salmon louse *Lepeophtheirus salmonis* and its toxicological and histopathological effects on Atlantic salmon *Salmo salar* and Chinook salmon *Oncorhynchus tshawytscha*. *Dis. Aquat. Organ.* 17, 197–204.
- Johnson, S.C., Treasurer, J.W., Bravo, S., Nagasawa, K., Kabata, Z., 2004. A review of the impact of parasitic copepods on marine aquaculture. *Zool. Stud.* 43, 229–243.
- Jones, M.W., Sommerville, C., Wootten, R., 1992. Reduced sensitivity of the salmon louse, *Lepeophtheirus salmonis*, to the organophosphate dichlorvos. *J. Fish Dis.* 15, 197–202.
- Jones, P.G., Hammell, K.L., Gettinby, G., Revie, C.W., 2013. Detection of emamectin benzoate tolerance emergence in different life stages of sea lice, *Lepeophtheirus salmonis*, on farmed Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* 36, 209–220.
- Lees, F., Baillie, M., Gettinby, G., Revie, C.W., 2008a. The efficacy of emamectin benzoate against infestations of *Lepeophtheirus salmonis* on farmed Atlantic salmon (*Salmo salar* L.) in Scotland, 2002–2006. *PLoS ONE* 3, e1549.
- Lees, F., Baillie, M., Gettinby, G., Revie, C.W., 2008b. Factors associated with changing efficacy of emamectin benzoate against infestations of *Lepeophtheirus salmonis* on Scottish salmon farms. *J. Fish Dis.* 31, 947–951.
- McAndrew, K.J., Sommerville, C., Wootten, R., Bron, J.E., 1998. The effects of hydrogen peroxide treatment on different life-cycle stages of the salmon louse, *Lepeophtheirus salmonis* (Krøyer, 1837). *J. Fish Dis.* 21, 221–228.
- Nakamura, K., Kanno, T., Mokudai, T., Iwasawa, A., Niwano, Y., Kohno, M., 2012. Microbial resistance in relation to catalase activity to oxidative stress induced by photolysis of hydrogen peroxide. *Microbiol. Immunol.* 56, 48–55.
- Nordberg, J., Arner, E.S.J., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.* 31, 1287–1312.
- Norwegian Institute of Public Health, 2014. Wholesaler-based Drug Statistics. <http://www.fhi.no/artikler/?id=109432> (accessed 20.10.14).
- Norwegian Medicines Agency, 2014. Summary of Product Characteristics for Paramove. <http://www.legemiddelverket.no/layouts/Preparatomtaler/Sp/11-8486.pdf> (accessed 20.10.14).
- Pike, A.W., Wadsworth, S.L., 2000. Sealice on salmonids: their biology and control. *Adv. Parasitol.* 44, 233–337.
- Robertson, J.L., Savin, N.E., Preisler, H.K., Russel, R.M., 2007. *Bioassays in Arthropods*, 2nd ed. CMC Press, New York.
- Roth, M., 2000. The availability and use of chemotherapeutic sea lice control products. *Contrib. Zool.* 69, 109–118.
- Roth, M., Richards, R.H., Dobson, D.P., Rae, G.H., 1996. Field trials on the efficacy of the organophosphorus compound azamethiphos for the control of sea lice (Copepoda: Caligidae) infestations of farmed Atlantic salmon (*Salmo salar*). *Aquaculture* 140, 217–239.
- Sevatdal, S., Copley, L., Wallace, C., Jackson, D., Horsberg, T.E., 2005. Monitoring of the sensitivity of sea lice (*Lepeophtheirus salmonis*) to pyrethroids in Norway, Ireland and Scotland using bioassays and probit modelling. *Aquaculture* 244, 19–27.
- Sevatdal, S., Horsberg, T.E., 2003. Determination of reduced sensitivity in sea lice (*Lepeophtheirus salmonis* Krøyer) against the pyrethroid deltamethrin using bioassays and probit modelling. *Aquaculture* 218, 21–31.
- Spitz, D.R., Adams, D.T., Sherman, C.M., Roberts, R.J., 1992. Mechanisms of cellular resistance to hydrogen peroxide, hyperoxia, and 4-hydroxy-2-nonenal toxicity – the significance of increased catalase activity in H₂O₂-resistant fibroblasts. *Arch. Biochem. Biophys.* 292, 221–227.
- Thomassen, J.M., 1993. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinneheim, K. (Eds.), *Fish Farming Technology*. Balkema, Rotterdam, pp. 233–236.
- Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O.T., Nilsen, F., Horsberg, T.E., Jackson, D., 2013. Salmon lice – impact on wild salmonids and salmon aquaculture. *J. Fish Dis.* 36, 171–194.
- Treasurer, J.W., Grant, A., 1997. The efficacy of hydrogen peroxide for the treatment of farmed Atlantic salmon, *Salmo salar* L. infested with sea lice (Copepoda: Caligidae). *Aquaculture* 148, 265–275.
- Treasurer, J.W., Wadsworth, S., Grant, A., 2000. Resistance of sea lice, *Lepeophtheirus salmonis* (Krøyer), to hydrogen peroxide on farmed Atlantic salmon, *Salmo salar* L. *Aquac. Res.* 31, 855–860.
- Uhlich, G.A., 2009. KatP contributes to OxyR-regulated hydrogen peroxide resistance in *Escherichia coli* serotype O157:H7. *Microbiology* 155, 3589–3598.
- Westcott, J.D., Hammell, K.L., Burka, J.F., 2004. Sea lice treatments, management practices and sea lice sampling methods on Atlantic salmon farms in the Bay of Fundy, New Brunswick, Canada. *Aquac. Res.* 35, 784–792.
- Westcott, J.D., Stryhn, H., Burka, J.F., Hammell, K.L., 2008. Optimization and field use of a bioassay to monitor sea lice *Lepeophtheirus salmonis* sensitivity to emamectin benzoate. *Dis. Aquat. Organ.* 79, 119–131.
- Whyte, S.K., Westcott, J.D., Elmoslemany, A., Hammell, K.L., Revie, C.W., 2013. A fixed-dose approach to conducting emamectin benzoate tolerance assessments on field-collected sea lice, *Lepeophtheirus salmonis*. *J. Fish Dis.* 36, 283–292.

III

Mechanism behind resistance against the organophosphate azamethiphos in salmon lice (*Lepeophtheirus salmonis*)

Kiranpreet Kaur*, Kari Olli Helgesen, Marit Jørgensen Bakke, Tor Einar Horsberg

NMBU School of Veterinary Science, Sea Lice Research Centre, PO Box 8146 Dep.,

NO-0033 Oslo, Norway

*Corresponding author. E-mail: kiran.kaur@nmbu.no, phone number: +47 22597130.

Short title: Azamethiphos resistance in salmon lice

Abstract

Acetylcholinesterase (AChE) is the primary target for organophosphates (OP). Several mutations have been reported in AChE to be associated with the reduced sensitivity against OP in various arthropods. However, to the best of our knowledge, no such reports are available for *Lepeophtheirus salmonis*. Hence, in the present study, we aimed to determine the association of AChE(s) gene(s) with resistance against OP. We screened the AChE genes (*L. salmonis ace1a* and *ace1b*) in two salmon lice populations: one sensitive (n=5) and the other resistant (n=5) for azamethiphos, a commonly used OP in salmon farming. The screening led to the identification of a missense mutation *Phe362Tyr* in *L. salmonis ace1a*, (corresponding to *Phe331* in *Torpedo californica* AChE) in all the samples of the resistant population. We confirmed the potential role of the mutation, with reduced sensitivity against azamethiphos in *L. salmonis*, by screening for *Phe362Tyr* in 2 sensitive and 5 resistant strains. The significantly higher frequency of the mutant allele (*362Tyr*) in the resistant strains clearly indicated the possible association of *Phe362Tyr* mutation in *L. salmonis ace1a* with resistance towards azamethiphos. The 3D modelling, short term survival experiments and enzymatic assays further supported the imperative role of *Phe362Tyr* in reduced sensitivity of *L. salmonis* for azamethiphos. Based on all these observations, the present study, for the first time, presents the mechanism of resistance in *L. salmonis* against azamethiphos. In addition, we developed a rapid diagnostic tool for the high throughput screening of *Phe362Tyr* mutation using High Resolution Melt analysis.

Introduction

Acetylcholinesterase (AChE), encoded by *ace* genes, is a serine hydrolase that plays a critical role in neurotransmission at cholinergic synapses and neuromuscular junctions. AChE is a target for two main classes of anti-cholinergic agents, organophosphates (OP) and carbamates (CB). OP and CB bind to the active site of AChE, and inactivate the enzyme by phosphorylating or carbamylating a serine residue in the enzyme's catalytic center [1]. The binding blocks the cleavage of the transmitter, acetyl choline (ACh), and results in elevated levels of ACh in the synaptic cleft thereby causing excitation, paralysis and death [2].

OPs have been used for treatment against salmon lice (*Lepeophtheirus salmonis*), a marine ectoparasitic copepod on salmonid species, in Norwegian salmonid aquaculture since the late 1970s. The first agent used was metrifonate (Neguvon), followed by dichlorvos (Nuvan) in 1986 and azamethiphos (Salmosan) in 1994 [3]. In 1991, the first cases of reduced efficacy of organophosphate treatments were noted in Mid-Norway [4]. When the use of azamethiphos was terminated during 1999, the problem of reduced sensitivity in salmon lice against azamethiphos was wide-spread. At that time, the cause of resistance was not determined.

Azamethiphos was re-introduced as a treatment agent against salmon lice in 2008 [5]. We received new reports of reduced efficacy of treatments with azamethiphos from the field in 2009. In 2013, a surveillance program, using bioassays to test for resistance, revealed a widespread distribution of azamethiphos resistance in Norwegian fish farms [5]. Bioassays are toxicological tests performed on live parasites and are thus labor intensive and associated with several sources of biases. Understanding the biochemical pathways underlying resistance in *L. salmonis* would therefore lead to the development of better tools to determine and control resistance. This would possibly improve management strategies and help in preventing economical loss due to ineffective treatments in the aquaculture industry.

Known resistance mechanisms towards organophosphates in arthropods include behavioral factors (the arthropod avoids the agent) and metabolic factors (e.g. enhanced activity of glutathion S-transferase or unspecific esterases) [6]. However, point mutations in AChE have been reported to be the most common mechanism behind reduced sensitivity in arthropods against OP [7].

Unfortunately, to the best of our knowledge, no study is available in the recent literature on AChE as a target site of OP in *L. salmonis*. We have recently identified and characterized the two genes coding for AChE in *L. salmonis* [8]. The full length cDNA sequences encoding the two AChEs in *L. salmonis* were identified and fully

characterized. Complete cDNA sequence encoding the *L. salmonis ace1a* (GenBank KJ132368) and *ace1b* (GenBank KJ132369) and the deduced amino acid sequences were determined. The two AChEs were highly similar to each other (84 % similarity at protein level), an observation quite unique to *L. salmonis* and has not been observed in other arthropods previously. *Ace1a* was predominantly expressed in different developmental stages of salmon lice compared to *ace1b* and was active in the cephalothorax, indicating that *ace1a* plays the major role in synaptic transmission [8].

In the present study, we aimed to determine the cause of reduced sensitivity in salmon lice against azamethiphos. This was achieved by screening the two acetylcholinesterase genes (*ace1a* and *ace1b*), in both sensitive and resistant *L. salmonis* populations. In addition, the effect of changes identified, on the expression, protein structure, activity of AChE and finally the survival of *L. salmonis* was also investigated and accomplished.

Materials and methods

Salmon lice strains and phenotypic characterization

Salmon lice samples were collected in the field. Four strains were kept in continuous culture [9] in the laboratory at The Norwegian Institute for Water Research's Marine Research Station at Solbergstrand, Drøbak (NIVA) or at the Institute of Biology, University of Bergen (UiB). The fish were anesthetized for handling procedures using Finquel vet (tricain mesilat, Western Chemical Inc., USA) dissolved in fresh water at final concentration of 125 mgL⁻¹ sea water. The fish were sacrificed in an anesthesia bath containing an overdose of the same substance. The Atlantic salmon applied as parasitic hosts at NIVA came from the commercial supplier Sørsmolt in Kragerø, Norway, while the rainbow trout came from the Norwegian University of Life Sciences (UMB) at Ås, Norway. The Atlantic salmon at UiB came from the breeding station of the Institute of Marine Research at Matre, Norway.

To characterize the salmon lice strains with regard to their sensitivity to azamethiphos, small scale treatments of fish infested with salmon lice were performed. In addition the sensitivity was tested by performing biological assays (bioassays) on salmon lice detached from the fish. The different strains of salmon lice included in the current study (Ls A, Ls G, Ls B, Ls H, Ls H-s, Ls V, Ls F and Ls 1998) are presented in Table 1 with their treatment history prior to collection, whether small scale treatments have been performed, and which type of bioassays have been performed.

The small scale treatments for efficacy evaluation were performed by treating one group of Atlantic salmon or rainbow trout infested with preadult parasites, of the salmon lice strain to be tested, with Salmosan (50 % w/w azamethiphos, Fish Vet Group, UK) at a concentration of 0.1 mgL^{-1} azamethiphos and keeping a separate group as untreated controls. The water exchange in the tanks was stopped for 30 minutes before the solution was drained and the tank rapidly refilled. The water was oxygenated during the treatment. The treatment effect was evaluated 5 - 7 days post treatment by counting parasites in both treatment and control groups. The first small scale treatment of Ls A and the small scale treatment of Ls H was performed at NIVA on rainbow trout and the results have been reported previously [10]. The second treatment of fish infested with Ls A was performed at UiB treating 6 Atlantic salmon weighing 300 grams each and keeping 6 fish as untreated control in another 500 liter tank. The fish had initially been infested with salmon lice in a common infestation trial. The effect of the treatment was evaluated 5 days post treatment.

The small scale treatment for genotype identification was performed on Ls F to see what genotypes that died at different time intervals during and after treatment. It was performed at NIVA on three salmon weighing 100 grams each which were infested with Ls F. The treatment was performed in a 100 liter tank, the same way as the other small scale treatments, but without a control group. During the exposure period and the first 2.5 hours thereafter, all sea lice detached from the fish were picked out of the tank and put on RNA-later. 24 hours post exposure all detached lice were removed from the tank and put on RNA-later. Seven days later all remaining sea lice were removed from the

fish and put on RNA-later. Three parasites had detached from the fish, but were alive and attached to the wall of the tank at 2 hours after initiation of the treatment. These were excluded from the analysis. After the experiments the fish were sacrificed in a lethal anaesthesia bath.

The sensitivities of the lab-cultivated strains towards azamethiphos were characterized by two types of bioassays and small scale treatments.

The 60 minute bioassay was performed on Ls A, Ls G, Ls B, Ls H and Ls V as described by Helgesen and Horsberg [10]. The results from Ls A and Ls H have been remodeled from data presented in Helgesen and Horsberg [10]. Preadult parasites were exposed to six different concentrations of azamethiphos (0-140 μgL^{-1} , different concentrations in different assays) in polystyrene boxes. After 60 minutes exposure, the boxes containing the parasites were rinsed in fresh sea water and kept in clean, aerated sea water at 12 °C for 24 hours before the parasites were characterized as either alive or dead/immobilized [10].

The 24 hour bioassays were performed on Ls A, Ls G, Ls H and Ls F by exposing preadult parasites to six different concentrations of azamethiphos (0-2 μgL^{-1} , different concentrations in different assays) in sea water for 24 hours, using glass bottles kept at 12 °C with constant aeration [10]. The results were read after 24 hours exposure by gently turning the bottles and thereafter pouring the solution into a beaker. Parasites attached to the bottle wall as well as parasites able to attach to the beaker wall or swim in a straight line, were characterized as alive. All other parasites in the beaker were categorized as dead/immobilized. The results from Ls A and Ls H has previously been presented in Helgesen and Horsberg [10].

Frozen samples (both surviving and immobilized parasites, n=9) from a bioassay performed in 1998 (a not given concentration of azamethiphos) were also enrolled in the study.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using RNeasy plus Mini kit (Qiagen, CA, USA), from female adult individuals, as per manufacturer's protocol. The RNA was quantified and qualified on ND-100 Spectrophotometer (Thermo Fisher Scientific, DE, USA). First strand cDNA was synthesized from total RNA (1 µg) using qScript reverse transcriptase (Quanta Biosciences, MD, USA).

*Screening of full length *L. salmonis ace1a* and *ace1b**

Full length cDNAs (*ace1a* and *ace1b*), from 5 sensitive (Ls A) and 5 resistant (Ls H) adult female sea lice samples, were amplified using gene specific primers (mentioned below). PCR reactions were performed using Phusion high-fidelity DNA polymerase (New England BioLabs, MA, USA) under the conditions: 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 55 °C for 15 s, 72 °C for 2 min followed by a final extension at 72 °C for 10 min. Amplicons were then subjected to direct sequencing using BIG Dye Terminator v3.1 cycle sequencing kit (Life technologies, Invitrogen, CA, USA) on 3130xl Genetic Analyzer (ABI Prism, Life technologies, Invitrogen, CA, USA).

Primers used to amplify the whole cDNA

ace1a forward primer: CTCTGCTGCTACACCGACTCCTGTT

ace1a reverse primer: TCGAGGATGTTTGACACTGATGGTC

ace1b forward primer: TGTTTTAGATGTGGATTCAAGTCCGAA

ace1b reverse primer: CGATGGATGGTACGTACGTATGAACATA

*Screening of missense changes identified in *L. salmonis ace1a* and *ace1b**

50 adult female samples each from 2 sensitive (Ls A and Ls G) and 2 resistant populations (Ls H, Ls B) were screened by direct sequencing. In addition, 2 *L. salmonis* populations (24 samples of Ls H-s and 20 samples of Ls V) that survived the azamethiphos treatment were also screened for these missense changes by direct sequencing.

Primers to amplify missense change in *L. salmonis ace1a*

ace1a forward primer: GTGGATGGAAGTTTCTTGGATGAGAG

ace1a reverse primer: CTCAAAGTTATTGCCTCTCTTCCCATT

Primers to amplify missense change in *ace1b*

ace1b forward primer: ACGAGCAAAGTCAGCAGTTG

ace1b reverse primer: TTTCATCCGCAGTGTTTCAG

Genotyping

The *Phe362Tyr* mutation in *L. salmonis ace1a*, which corresponds to codon 331 in the *Torpedo californica* AChE, was validated by High Resolution Melt (HRM) analyses, which is a simple rapid tool to screen single base changes (mutations/polymorphisms) with high sensitivity and accuracy [11]. The methodology included the generation of specific PCR product using gene specific primers (mentioned below) and Precision Melt supermix (Bio Rad, CA, USA), as per manufacturer's instructions, with a sensitive fluorescent dye (EvaGreen) that binds specifically only to double stranded DNA, followed by subjecting the amplicon to gradual increase in temperature (65 °C to 95 °C), which led to the denaturation of double stranded amplicon and decrease in fluorescence. This change in fluorescence was recorded by the C1000 Touch thermal cycler (Bio-Rad, CA, USA) as a melt curve (fluorescence versus temperature). The samples were assembled into different groups based on difference in the shapes of their melt curves.

HRM primers for the *Phe362Tyr* mutation

Forward primer: TTTTAATTGGAGCGAATAAGGA

Reverse Primer: TCTGTTTCGATCAACATAGACG

The typing of parasites from the small scale treatment for genotype identification were performed by qPCR using TaqMan probes specific for the sensitive (S) and resistant (R) genotypes. The assay was developed for high throughput analyses by PatoGen Analyse AS, based on the results presented here. By combining the probes, each

parasite could be classified as sensitive (SS), heterozygote (RS) or homozygote resistant (RR). Genotyping could be performed in all except one parasite that was dead at 24 hours after initiation of the treatment.

Alignment of amino acid sequences

Deduced amino acid sequences of *L. salmonis* AChE1a and AChE1b were compared with 33 previously published AChE protein sequences from other species, using CLUSTALW program with BLOSUM matrix and default settings [12] to obtain Multiple sequence alignment (MSA).

3D modelling of the enzymes

The three-dimensional structure of the AChE1a enzyme from *L. salmonis* was modeled using SWISS MODEL in the automated mode, (<http://swissmodel.expasy.org/>) [13]. An initial template search using the wild-type AChE1a from *L. salmonis* as target revealed several possible templates. The best fit was found with native AChE from *D. melanogaster*, PDB-ID 1qo9 [14] (RMS 0.25 for the whole protein, 0.05 for ten amino acids important for choline binding, the catalytic triad, the acyl pocket and the oxyanion hole). The Root Mean Square (RMS) for the fit between template and target were calculated using the Swiss PDB viewer 4.1.0. (<http://www.expasy.org/spdbv/>). Azamethiphos was docked to the wild-type and the mutated AChE1a using the online molecular docking server (<http://www.dockingserver.com/web>) and the best fit was illustrated using the UCSF Chimera 1.10.1. software (<http://www.cgl.ucsf.edu/chimera/>).

Inhibition of enzymatic activity

Two approaches were used to assess inhibition of AChE activity and possible differences between OP-susceptible and -resistant *L. salmonis* strains. In both the experiments, only preadult females were used. Lice from the susceptible strain (Ls A) were all expected to be SS. This was based on the frequency of the genotype SS (100 %) in the screening of this strain. All the samples from the resistant strain were individually cut in two with a sterile scalpel. One half was put in RNA *later* for

subsequent genotyping, whereas its counterpart was stored at -80 °C for enzymatic assay. This allowed the use of only confirmed RR-lice in the assays.

In vitro treatment

To assess the importance of the *Phe362Tyr* mutation (corresponds to Phe331 in *Torpedo californica* AChE) *in vitro*, a slightly modified version of a protocol developed by the World Health Organization (WHO) to detect insecticide resistance mechanisms in mosquitoes (WHO/CDS/CPC/MAL/98.6) was used [15]. The modifications were needed to optimize the protocol for sea lice. In brief, samples (one whole or two half preadult II/ two whole or four half preadult I) were homogenized in 75 µl deionized (18 MΩ) water with a pestle. To reduce the influence of protease activity, the samples were prepared just a few minutes before the assay was started and were kept on ice at all times. The enzymatic activity was analyzed using the principle of Ellman *et al.* (1961) on 96 wells microtitre plates [16]. The wells contained phosphate buffer (0.1 M, pH 7.8) with 1 % Triton X-100 (140 µl), 5,5'-Dithiobis-(2-nitrobenzoic acid) in phosphate buffer (10 mM work solution; 10 µl) and 10 mM acetylthiocholine iodide (ATC) in deionized water (10 mM work solution; 25 µl). In a parallel series, propoxur (0.1 M in acetone) was added to the ATC work solution giving a concentration of 0.2 mM propoxur. The ingredients in the wells were gently mixed before the addition of lice homogenate (25 µl) to both parallels. This allowed for comparison of AChE activity with and without propoxur inhibition (n=21 for RR and n=20 for SS). The microtitre plates were shaken for 1 minute and immediately read in kinetic mode for 10 minutes at 405 nm (Epoch spectrophotometer, BioTek, USA). The individual slopes were calculated based on the best linear fit (Gen5 version 2.00) and a standard curve prepared from AChE from electric eel (SigmaAldrich) was used to calculate the enzymatic activity. One unit (U) is the amount of enzyme expected to catalyze 1 µmole substrate per minute. For each sample the activity is expressed relative to the protein content in the homogenate (Umg⁻¹ protein). Protein content was measured on a Take3 plate in an Epoch spectrophotometer (BioTek Instruments Inc., USA) and calculated in Gen5 version 2.0 using a build-in standard curve for bovine serum albumin (BioTek Instruments Inc., USA). Inhibition in percent of normal AChE activity was calculated for each sample (=100-(activity with propoxur*100)/ activity without propoxur).

In vivo treatment

Preadult female lice were collected alive and randomly assigned to either a 0 μgL^{-1} or 2 μgL^{-1} azamethiphos bath exposure for 24 hours. The exposures were carried out on the detached parasites in filtered and continuously aerated sea water in 1 liter glass bottles kept at 10 °C. Susceptible and resistant strains were kept in separate bottles. After 24 hours the lice were collected and sampled as described above. Residual AChE activity was measured following the modified WHO protocol (see *in vitro* section). Because azamethiphos was used to block AChE activity, no propoxur was added to the mixtures. AChE activity could not be measured before and after treatment in the same individual, hence the results are presented as absolute values instead of relative values.

Statistics

All bioassay results were modelled using probit analysis in the statistical software JMP 10 (SAS Institute Inc., Cary, NC, USA) and EC_{50} -values (the concentration immobilizing 50 % of the parasites) with 95 percent confidential intervals were calculated. The mean treatment efficacies in the small scale treatments were calculated using bootstrapping with 2500 simulations calculating the difference (in %) of parasites between the treated groups and their respective control groups using JMP. The 95 % confidence intervals (CI) for efficacy were constructed using the number of fish per treated group as N.

An analysis of survival versus time for the three genotypes was then conducted with a Kaplan-Meier survival analysis including a Wilcoxon test (JMP).

The results from the enzymatic inhibition studies were statistically compared (JMP) with ANOVA after root transformation (*in vitro* study) or the non-parametric multiple comparison Steel-Dwass method (*in vivo* study).

Ethics Statement

The studies were approved by the NIVA local ethics committee, ID 2995, in accordance with the guidelines set by The Norwegian Animal Research Authority. The research

station is approved as a fish research facility by the Norwegian Animal Research Authority.

Results

Bioassays and small scale treatment for phenotypic characterization

All results from small scale treatments and bioassays, performed to characterize the salmon lice strains with regard to their sensitivity to azamethiphos, are given in Table 2. Ls A showed high mortalities in the two small scale treatments, 100 % and 98 %, respectively. Both Ls A and Ls G showed low EC₅₀-values (< 3 µgL⁻¹ in the 60-minutes and < 0.2 µgL⁻¹ in the 24-hour bioassays). Fifty percent of the sea lice from Ls H died in the small scale treatment. The EC₅₀-values (60-minutes assay) from this strain was more than 28 times higher than the values from Ls A and Ls G. The EC₅₀-value (60-minutes assay) from Ls B was higher than the corresponding value in the 60-minutes assay on Ls A but much lower than the Ls H EC₅₀-values from the 60-minutes bioassay. The strain used in the small scale treatment experiment for genotype identification, Ls F, demonstrated sensitivity that was lower than Ls A and Ls H in a 24-hour bioassay.

*Screening of the *L. salmonis ace1a* and *ace1b* genes for polymorphisms*

The screening of whole cDNA sequence of both the genes in five sensitive (Ls A) and five resistant (Ls H) salmon lice revealed one non-synonymous change and two silent changes in *L. salmonis ace1a*. The non-synonymous change led to an amino acid change: phenylalanine to tyrosine at codon 362, which corresponds to codon 331 in the *Torpedo californica* amino acid sequence. The two other substitutions were silent changes (Fig. 1).

In *ace1b*, a single change was identified in codon 433, leading to Isoleucine -> Threonine substitution (Fig. 2), which corresponds to codon Ile401 in the *T. californica* amino acid sequence. The frequencies of this change is listed in the supporting information, S1 Table.

Association of missense changes in ace1a and ace1b with resistance against azamethiphos

Both the non-synonymous changes (*Phe362Tyr* in *ace1a* and *Ile433Thr* in *ace1b*) were screened, by direct sequencing, in laboratory cultured sea lice populations, including the two sensitive strains (Ls A, Ls G) and the two strains with reduced sensitivity (Ls B, Ls H) to determine their association with resistance against azamethiphos. Fifty samples from each population were enrolled for screening. None of these populations were under any treatment pressure when enrolled. In addition, 20 parasites that survived a normal field treatment with azamethiphos (Ls V) along with 24 samples from Ls H surviving a small scale azamethiphos treatment were also screened for the *Phe362Tyr* change. The results (Table 3) demonstrated a clear association between the sensitivity classification and the frequency of the *Phe362Tyr* mutation. However, no such association was observed for the *Ile433Thr* change in *L. salmonis ace1b* and the sensitivity classification (S1 Table).

Phe362Tyr in samples collected in 1998

The salmon lice samples (n=9) after the selection experiment with azamethiphos in 1998 were also screened for the *Phe362Tyr* change. The screening revealed that this change was present in all the salmon lice (n=4) that survived the exposure (SS=0, RS=3 and RR=1) at that time. None of the samples that died during azamethiphos exposure harbored the mutation (n=5).

Phe362Tyr

The alignment of the *L. salmonis* AChE1a protein with 33 AChE amino acid sequences from other species revealed that the *Phe362Tyr* in *ace1a* is homologous to *Phe331* of AChE in *T. californica* and is located in the acyl pocket neighboring the catalytic center in the active site gorge. It is a highly conserved residue among the species as evident from multiple sequence alignment (MSA) of AChEs from different species (Fig. 3).

3D modelling of the enzyme

The 3D modeling was performed using SWISS MODEL <http://swissmodel.expasy.org/> [13], <http://swissmodel.expasy.org/>. The 3D structure of the native enzyme from *Drosophila melanogaster* (PDB ID: 1qo9) was used as a template. The protein from *L. salmonis* could fit the template, but the fit was not optimal. The QMEAN4 score (a parameter between 0 and 1 where a higher number indicates a better fit) was 0.541. However, the Root Mean Square (RMS) values were low, 0.25 for the whole protein and 0.05 for ten essential amino acids. Thus, the models were still considered useful.

The generated pdb files are included in the supplementary material (S1 file and S2 file).

The 3D model (Fig. 4) revealed that the change to *Tyr* at position 362, resulted in interference with the entrance to the catalytic triad of the enzyme (*Ser230*, *Glu358* and *His472* in *L. salmonis*, corresponding to *Ser200*, *Glu327* and *His440* in *T. californica*). The aromatic ring of *Tyr* is turned approximately 50 degrees compared to the aromatic ring of *Phe*. Tyrosine has a hydroxyl group in the para-position, which enters the groove leading to the catalytic triad, decreasing the volume of the pocket. The substitution of the nonpolar *Phe* with the polar *Tyr* also changes the polarity of the active gorge and thereby the binding site for organophosphates, most likely affecting binding of these molecules in the enzyme. The best fit of azamethiphos in the catalytic gorge of the wild-type and in the mutated enzyme implied hydrogen (H) bonds between 362*Tyr* and azamethiphos, and between *Tyr152* and azamethiphos. The model did not predict H-bonds between *Phe362* and azamethiphos, or between *Tyr152* and azamethiphos in the wild-type enzyme.

High Resolution Melt analysis (HRM)

High Resolution Melt analysis (HRM) was performed to validate the sequencing results and in an attempt to develop a rapid diagnostic tool for the detection of *Phe362Tyr* mutation in *L. salmonis ace1a*. After standardizing the technique with samples of known genotypes, determined by direct sequencing (wild type, heterozygous and homozygous for *Phe362Tyr* mutation), samples with unknown genotypes were run to confirm the

results obtained. These were also confirmed by direct sequencing. HRM analysis could distinguish between the samples of different genotypes with high accuracy. As shown in Fig. 5, the samples were very well separated based on their genotypes.

Treatment trial for genetic characterization

The frequency of live and dead parasites from Ls F within each genotype is given in Table 4. All parasites of the SS genotype died within two hours after initiation of the treatment, while no parasites of the RR genotype died within eight days after the treatment. The mortality rate of the RS genotype was 44 %.

The Kaplan-Meier survival analysis of the SS genotype demonstrated that the median survival time was 25 minutes (95 % CI: 15 - 30 min). A Wilcoxon test showed highly significant differences between the groups ($\chi^2=64.7$, DF=2, $p < 0.0001$). The survival plot is displayed in Fig. 6.

Enzyme inhibition assay

The degree of enzymatic inhibition was assessed both *in vitro* (with propoxur added to lice homogenates) and *in vivo* (with azamethiphos exposure of live salmon lice). One azamethiphos-susceptible and one -resistant strain of parasites were used, representing the genotypes SS and RR, respectively.

In the *in vitro* assay, residual activity was calculated in homogenates with propoxur added to the wells and normalized to the activity in the non-inhibited fraction. A statistically significant difference was found in residual activity between the two genotypes SS and RR ($p < 0.0001$). The arithmetic means of the residual activities were 21.1 % and 37.3 % with confidence intervals (95 %) of [16.1; 26.2] and [32.4; 42.2] for SS and RR, respectively (Fig. 7).

Inhibition with azamethiphos was done on live lice (*in vivo*); hence one louse could only belong to one of the treatment groups. In contrast to the *in vitro* experiments, residual activity could therefore not be normalized. Instead, the absolute values of enzyme

activity were used to compare the four groups (Fig 8). Azamethiphos, $2 \mu\text{gL}^{-1}$, inhibited AChE activity in both the susceptible strain (SS; $p < 0.0001$) and the resistant strain (RR; $p < 0.0001$). In the susceptible strain (SS) the median activities were $61.6 \text{ mU/mg protein}$ ($0 \mu\text{gL}^{-1}$) and $7.0 \text{ mU/mg protein}$ ($2 \mu\text{gL}^{-1}$) and in the resistant strain (RR) the median activities were $72.7 \text{ mU/mg protein}$ ($0 \mu\text{gL}^{-1}$) and $16.9 \text{ mU/mg protein}$ ($2 \mu\text{gL}^{-1}$), respectively. No statistically significant difference was found between SS and RR control groups (not exposed to azamethiphos). After exposure to $2 \mu\text{gL}^{-1}$ azamethiphos, there was a significant difference in absolute residual activity between the two genotypes ($p = 0.019$), indicating that the *L. salmonis* mutation *Phe362Tyr* is involved in the protection against azamethiphos. All the lice in the SS- $2 \mu\text{gL}^{-1}$ group were immobilized when sampled. In the RR- $2 \mu\text{gL}^{-1}$ group, the behavior was not notably different from the control group (RR- $0 \mu\text{gL}^{-1}$), as only 6.8 % and 4.7 % were immobilized at the end of the observation period, respectively. The AChE activities in all of the samples from the SS group were below the median value in the RR group.

Discussion

Decreased sensitivity for various chemotherapeutics has become a major issue in controlling the sea lice problem worldwide with Norway being no exception [5]. Azamethiphos has been one of the most commonly used chemical treatment agents against sea lice in Norway for decades [3]. However, the development of resistance over the years, attributed to its overuse, has affected the fish farm industry a lot. Unfortunately, no tool is yet available to identify resistance because of the lack of knowledge about the molecular mechanisms involved in resistance.

As per the existing literature, resistance towards azamethiphos is mostly associated with mutations in AChE genes in various arthropods [7]. Among these, point mutations are the most commonly found mutations [7]. Around 70 different mutations have been reported in AChE genes to be associated with decreased sensitivity against azamethiphos in various species [7]. Majority of these missense mutations have been found in or around the active gorge site of the enzyme, making it a hot spot for mutations [7].

In the present study, we investigated the molecular mechanisms of azamethiphos resistance in *L. salmonis*. Based on our results we could substantially state that *L. salmonis ace1a* is the primary target for azamethiphos and that the *Phe362Tyr* replacement (corresponding to *Phe331* in *T. californica*) is primarily responsible for conferring reduced sensitivity in *L. salmonis* against azamethiphos.

Phe362 position in *L. salmonis* is homologous to *Phe331* position in *T. californica* and is located in the acyl pocket neighboring the active centre in the active site gorge (Fig. 4) [17]. The acyl pocket is responsible for ligand specificity via two properties. The first property is related to the formation of the enzyme-substrate intermediate complex. The acyl pocket is located at the bottom of the active site and surrounded by side chains of hydrophobic aromatic residues. Because of its location and surroundings, acyl pocket attracts and orientates the acyl group of the substrate and inhibitors through its hydrophobicity during the catalytic reaction [17, 18].

The second property of the acyl pocket derives from its electrostatic field. In *T. californica*, the pocket forming the side chain of *Phe331* attracts the catalytic *His440* by cation- π interaction. *Phe331* is considered to arrange the catalytic histidine so that proper conformational change of the histidine can occur in the hydrolyzing step. Mutagenesis studies followed by computer simulation demonstrated that the orientation of *His447* in human AChE (corresponds to *His440* in *T. californica*) is changed by substitution from the wild type *Phe338* (*Phe331* in *T. californica*) to aliphatic residues [19, 20]. However, whether a substitution with Tyr at this position affects the cation- π interaction is not known. Nevertheless, this substitution in the *L. salmonis* AChE1a is considered to affect the inhibitor enzyme interaction either by changes in inhibitor affinity or interaction with the catalytic histidine, or both.

In the present study, the 3D model of the AChE1a in *L. salmonis* revealed that the *Phe362Tyr* substitution makes the acyl pocket smaller and more polar (Fig. 4), which could alter the accessibility of azamethiphos to the site. The docking of azamethiphos to the enzyme also suggested that both *362Tyr* and *Tyr152* formed H-bonds with

azamethiphos in the mutated enzyme, thereby interfering with the capability of azamethiphos to bind to serine in position 230 (*Ser200* in *T. californica*). In addition, the mutation screening revealed a significantly higher frequency of *362Tyr* in resistant samples compared to the sensitive samples (Table 3). Moreover, all the survivors of azamethiphos treatment of the Ls H strain, (Ls H-s) carried *362Tyr* mutant allele (Table 3); increasing the frequency of *362Tyr* from 56 % to 100 % (92 % samples with one mutant allele (RS) and 8 % samples with both the mutant alleles (RR)). This clearly indicates the importance of *362Tyr* in the survival of sea lice under azamethiphos exposure.

The association between the mutation *Phe362Tyr* and salmon lice resistance against OPs was further supported by enzymatic assays of AChE activity using the two inhibitors (propoxur and azamethiphos) in one *in vitro* and one *in vivo* assay, respectively. Propoxur inhibited AChE activity at a significantly lower degree in resistant *L. salmonis* with the *Phe362Tyr* mutation (RR) compared to salmon lice without the mutation (SS). This is in accordance with a standardized assay developed by the World Health Organization (WHO) to assess insecticide resistance in mosquitoes [15] although the cut-off for classifying resistance in mosquitos does not apply to salmon lice. An important reason for this is the presence of two AChEs in salmon lice [8]. The assay was done on homogenate of whole louse, thus both AChE1a and AChE1b contributed to the total enzymatic activity measured. However, the relative contribution between the two AChEs and their significance for the salmon lice to survive has not yet been clarified. In mosquitos only one AChE has been characterized. Therefore, a resistance-related mutation would be significantly more effective in preventing propoxur inhibition, as shown in *Anopheles subpictus* [21], *Anopheles maculipennis* [22] and *Culex quinquefasciatus* [23]. The *in vitro* results suggest a substantial contribution of AChE1b to the total AChE-activity in *L. salmonis*. As no resistance-associated mutations were found in AChE1b, this enzyme was assumed to be fully inhibited by propoxur in both the SS and the RR group. Thus, it is the AChE1a residual activity in *362Tyr* samples compared to *Phe362* samples that renders the parasite capable of surviving an azamethiphos treatment. This was examined in the *in vivo experiment* and

ties the link between the biochemical effect alone and the survival after azamethiphos treatment. The results confirm that there was a difference in residual activity also after exposure of live parasites to azamethiphos. The somewhat greater dispersion of the data points in the RR group suggest that the relative contribution of AChE1a and AChE1b can vary between individuals. Thus, a cut-off limit for the total AChE activity cannot be used as an indicator for the *Phe362Tyr* mutation until more knowledge on the different contribution of the two proteins, both quantitatively and qualitatively, has been generated.

The low frequency of *362Tyr* in the samples without azamethiphos treatment (Ls G, 4 %) could be explained by the theory suggested by Shi *et al.* (2004), which states that even though there is a fitness cost associated with mutations in AChE, conferring resistance towards OP, the alleles might still survive without selection pressure [24]. The frequency of the mutant allele in the natural population (without treatment pressure) depends on the alteration caused by mutant allele on the protein. The point mutations cause a low level of alteration in the protein, which is the main driving force responsible for the maintenance of resistant alleles in natural populations [24]. This theory is supported by the fact that most of the mutations reported in AChE are point mutations [7]. Further, the presence of *362Tyr* in the samples from 1998 is another evidence in support of the theory, as this ascertains the presence of mutant (*362Tyr*) allele in the salmon lice population, without selection pressure, for eight (2000-2007) years [25, 26].

Around 70 different missense mutations have been reported to be associated with OP resistance in *ace* genes from other species [7]. Interestingly, none of these missense mutations were found in the *ace1a* gene in our resistant samples, neither in the samples from 1998, nor in later samples. This observation suggests a single origin of *Phe362Tyr* mutation, which dispersed intensively due to the immense selection pressure caused by repeated OP treatments in salmon farms. As the first cases of OP resistance in salmon lice were reported in 1991 [4], it is most likely that the mutation was originated at that time.

The observations of the present study very well supported the theory by Shi *et al.* (2004) [24]. As the mutant allele could affect the fitness of the salmon lice (without azamethiphos treatment), the frequency of samples carrying *362Tyr* goes down (4 %) in the natural population (Ls G). However, the mutant allele (*362Tyr*) persists in the natural population along with the wild type allele (*Phe362*) as the fitness cost is limited. After treatment the frequency of *362Tyr* shoots up (100 % in Ls H-s, Table 3; of which 92 % carried one mutant allele and the remaining were homozygous for *362Tyr*) i.e. only the carriers of the mutant allele (*362Tyr*) survived the treatment.

The mutation screening experiment was further validated by the small scale treatment experiment (Fig. 6), which showed that all the samples without *Phe362Tyr* substitution (SS) died within 2 hours of the treatment whereas no mortality was seen among the homozygous samples (RR) for *362Tyr* substitution. 44 % of the samples that carried only one mutated allele (RS) died. This observation again indicated that *362Tyr* plays a vital role in the survival of salmon lice under azamethiphos treatment.

Phe331 (numbering from *T. californica*) is highly conserved among AChE1 and AChE2 from various species as shown in Fig. 3. The high conservation of *Phe331* and its location in AChE at an important site, signals its potential significance in the protein function and in turn the survival of organism (Fig. 3 and Fig. 4).

Together, all the observations of the present study, clearly point towards a strong association of the *Phe362Tyr* substitution in *L. salmonis* with decreased sensitivity of sea lice towards azamethiphos.

To the best of our knowledge, *Phe362Tyr* mutation (codon 331 in *T. californica*) has not been reported earlier. However, there are reports about other resistance-associated point mutations at the same amino acid position. For example, substitution of Ser with Phe in this position in AChE2 of *Myzus persicae* was found to be associated with insensitivity towards pirimicarb [27], and towards pirimicarb and omethoate in AChE1 of *Ashbya gossypii* (*Ser431Phe* in this species) [28]. Alon *et al.* (2008) have reported

Phe392Trp (*Phe331* in *T. californica*) substitution in AChE1 of *Bemisia tabaci* [29]. A similar mutation, *Phe455Trp* (*Phe331* in *T. californica*), was reported in AChE2 of *Culex tritaeniorhynchus*, in association with extreme insecticide insensitivity (30-fold) and was considered to be solely responsible for the insecticide –resistance of AChE in these mosquitoes [30, 31]. Expression of this mutation in AChE1 from *C. tritaeniorhynchus* in a baculovirus-Sf9 cell system and subsequent treatment of the expressed proteins with OP and carbamate inhibitors revealed extremely reduced sensitivity to OP compounds [31]. Anazawa *et al.* (2003) observed a 140 fold decrease in sensitivity towards the OP dichlorvos in *Tetranychus urticae* with a change from Phe to Cys at the same position [32]. Similarly, Kwon *et al.* (2012) found a 99 fold decrease in sensitivity to monocrotophos and a significant decrease in catalytic efficiency of the enzyme in *T. urticae* carrying a *Phe439Trp* (*Phe331Trp* in *T. californica*) mutation [33].

Mutagenesis studies with human AChE also demonstrated that *Phe338* (*Phe331* in *T. californica*) to Ala replacement conferred a 2-fold decrease in edrophonium-sensitivity [34].

Various studies involving the mutagenized and naturally occurring substitutions in insect AChEs have also inferred the importance of positions homologous to the *T. californica* *Phe331* position, for reduced sensitivity towards AChE-inhibiting insecticides. For example, an *in vitro* mutagenesis study carried out with *Drosophila melanogaster ace2*, demonstrated that substitutions of *Phe371* (homologous position in *D. melanogaster ace2* to *Phe331* in *T. californica*) to Ala, Gly, Ile and Tyr resulted in 10-100 fold decrease in carbamate sensitivity. Interestingly, a 100 fold decrease in carbaryl, malaoxon and paraoxon sensitivity with *Phe371Tyr* (*Phe331Tyr* in *T. californica*) substitution was observed by site-directed mutagenesis [35]. This engineered mutation is homologous to the natural mutation described in the current study and further strengthens the importance of *Phe331* in sensitivity towards OPs, as well as the significance of the described *Phe362Tyr* mutation in *L. salmonis*.

In conclusion, four lines of evidence for the significance of the *Phe362Tyr* (*L. salmonis*) mutation in relation to salmon lice resistance towards azamethiphos are presented here. Firstly, the significantly high frequency of *362Tyr* in *L. salmonis* samples resistant to azamethiphos indicated a clear association of *Phe362Tyr* with reduced sensitivity towards azamethiphos (Table 3). Secondly, the 3D modelling suggested that *362Tyr* could affect the access and binding of azamethiphos at the active site (Fig. 4). Thirdly, the treatment trial for genetic characterization with azamethiphos showed 0 % mortality in samples with both the mutated (*362Tyr*) alleles (Table 3). And finally the enzymatic assay revealed a significantly higher residual activity in resistant (RR) versus the sensitive (SS) samples (Fig. 7 and Fig. 8) both *in vitro* and *in vivo*. Taken together, all these observations provide a strong argument in favor of *Phe362Tyr* mutation being the culprit behind azamethiphos resistance in *L. salmonis*.

Acknowledgements

We would like to thank Stian Mørch Aaen for carrying out one of the small scale treatment on Ls A. We would also like to thank PatoGen Analyse AS for performing analyzes of the samples from the treatment trial for genetic characterization.

References

1. Aldridge WN. Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl p-nitrophenyl thiophosphate (E 605) and analogues. *Biochem J.* 1950;46: 451-460.
2. Fournier D, Mutero A. Modification of acetylcholinesterase as a mechanism of resistance to insecticides. *Comp Biochem Phys C.* 1994;108: 19-31.
3. Torrissen O, Jones S, Asche F, Guttormsen A, Skilbrei OT, Nilsen F et al. Salmon lice--impact on wild salmonids and salmon aquaculture. *J Fish Dis.* 2013; 36: 171-194.
4. Denholm I, Devine GJ, Horsberg TE, Sevatdal S, Fallang A, Nolan DV et al. Analysis and management of resistance to chemotherapeutants in salmon lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Pest Manag Sci.* 2002;58: 528-536.
5. Grøntvedt RN, Jansen PA, Horsberg TA, Helgesen K, Tarpai A. The surveillance programme for resistance to chemotherapeutants in *L. salmonis* in Norway. Surveillance programmes for terrestrial and aquatic animals in Norway. Annual report 2013. Oslo: Norwegian Veterinary Institute 2014.
6. Riaz MA, Chandor-Proust A, Dauphin-Villemant C, Poupardin R, Jones CM, Strode C et al. Molecular mechanisms associated with increased tolerance to the neonicotinoid insecticide imidacloprid in the dengue vector *Aedes aegypti*. *Aquat Toxicol.* 2013;126: 326-337.
7. Hotelier T, Nègre V, Marchot P, Chatonnet A. Insecticide resistance through mutations in cholinesterases or carboxylesterases: data mining in the ESTHER database. *J Pestic Sci.* 2010;35: 315-320.
8. Kaur K, Bakke MJ, Nilsen F, Horsberg TE. Identification and molecular characterization of two acetylcholinesterases from the salmon louse, *Lepeophtheirus salmonis*, PLOS ONE. 2015. In press
9. Hamre LA, Glover KA, Nilsen F. Establishment and characterisation of salmon louse (*Lepeophtheirus salmonis* (Krøyer 1837)) laboratory strains. *Parasitol Int.* 2009;58: 451-460.

10. Helgesen KO, Horsberg TE. Single-dose field bioassay for sensitivity testing in sea lice, *Lepeophtheirus salmonis*: development of a rapid diagnostic tool. J Fish Dis. 2013;36: 261-272.
11. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics 2007;8: 597-608.
12. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22: 4673-4680.
13. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. Bioinformatics 2006;22: 195-201.
14. Harel M, Kryger G, Rosenberry TL, Mallender WD, Lewis T, Fletcher RJ et al. Three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and of its complexes with two potent inhibitors. Protein Sci. 2000;9: 1063-1072.
15. WHO (World Health Organization). Techniques to detect insecticide resistance mechanisms (field and laboratory manual). WHO/ CDS/CPC/MAL/98.6. 1998
16. Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7: 88-95.
17. Hosea NA, Berman HA, Taylor P (1995) Specificity and orientation of trigonal carboxyl esters and tetrahedral alkylphosphonyl esters in cholinesterases. Biochemistry 1995;34: 11528-11536.
18. Ordentlich A, Barak D, Kronman C, Benschop HP, De Jong LP, Ariel N et al. Exploring the active center of human acetylcholinesterase with stereoisomers of an organophosphorus inhibitor with two chiral centers. Biochemistry 1999;38: 3055-3066.
19. Shafferman A, Ordentlich A, Barak D, Stein D, Ariel N, Velan B. Aging of phosphorylated human acetylcholinesterase: catalytic processes mediated by aromatic and polar residues of the active centre. Biochem J. 1996;318: 833-840.

20. Bar-On P, Millard CB, Harel M, Dvir H, Enz A, Sussman JL et al. Kinetic and structural studies on the interaction of cholinesterases with the anti-Alzheimer drug rivastigmine. *Biochemistry* 2002;41: 3555-3564.
21. Surendran SN, Jude PJ, Weeraratne TC, Parakrama Karunaratne SHP, Ramasamy R. Variations in susceptibility to common insecticides and resistance mechanisms among morphologically identified sibling species of the malaria vector *Anopheles subpictus* in Sri Lanka. *Parasit Vectors* 2012;5: 34.
22. Muhammet MA. Malathion and Propoxur Resistance in Turkish Populations of the *Anopheles maculipennis* Meigen (Diptera: Culicidae) and Relation to the Insensitive Acetylcholinesterase. *Turkiye Parazitoloj Derg.* 2014;38: 111-115.
23. Low VL, Chen CD, Lee HL, Tan TK, Chen CF, Leong CS et al. Enzymatic characterization of insecticide resistance mechanisms in field populations of Malaysian *Culex quinquefasciatus* Say (Diptera: Culicidae). *PLOS ONE* 2013;8: e79928.
24. Shi MA, Lougarre A, Alies C, Frémaux I, Tang ZH, Stojan J et al. (2004) Acetylcholinesterase alterations reveal the fitness cost of mutations conferring insecticide resistance. *BMC Evol Biol.* 2004;4: 5.
25. Grave K, Horsberg TE, Lunestad BT, Litleskare I. Consumption of drugs for sea lice infestations in Norwegian fish farms: methods for assessment of treatment patterns and treatment rate. *Dis Aquat Organ.* 2004;60: 123-131.
26. Helgesen KO, Bravo S, Sevatdal S, Mendoza J, Horsberg TE. Deltamethrin resistance in the sea louse *Caligus rogercresseyi* (Boxhall and Bravo) in Chile: bioassay results and usage data for antiparasitic agents with references to Norwegian conditions. *J Fish Dis* 2014;37: 877-890.
27. Nabeshima T, Kozaki T, Tomita T, Kono Y. An amino acid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, *Myzus persicae*. *Biochem Biophys Res Commun.* 2003;307: 15-22.
28. Benting J, Nauen R. Biochemical evidence that an S431F mutation in acetylcholinesterase-1 of *Aphis gossypii* mediates resistance to pirimicarb and omethoate. *Pest Manag Sci.* 2004;60: 1051-1055.
29. Alon M, Alon F, Nauen R, Morin S. Organophosphates' resistance in the

- B-biotype of *Bemisia tabaci* (Hemiptera: Aleyrodidae) is associated with a point mutation in an ace1-type acetylcholinesterase and overexpression of carboxylesterase. *Insect Biochem Mol Biol.* 2008;38: 940-949.
30. Nabeshima T, Mori A, Kozaki T, Iwata Y, Hidoh O, Harada S et al. An amino acid substitution attributable to insecticide-insensitivity of acetylcholinesterase in a Japanese encephalitis vector mosquito, *Culex tritaeniorhynchus*. *Biochem Biophys Res Commun* 2004;313: 794-801.
 31. Oh S-H, Kozaki T, Mizuno H, Tomita T, Kono Y (2006) Expression of Ace-paralogous acetylcholinesterase of *Culex tritaeniorhynchus* with an amino acid substitution conferring insecticide insensitivity in baculovirus-insect cell system. *Pestic Biochem Phys* 2006;85: 46-51.
 32. Anazawa Y, Tomita T, Aiki Y, Kozaki T, Kono Y. Sequence of a cDNA encoding acetylcholinesterase from susceptible and resistant two-spotted spider mite, *Tetranychus urticae*. *Insect Biochem Mol Biol.* 2003;33: 509-514.
 33. Kwon DH, Choi JY, Je YH, Lee SH. The overexpression of acetylcholinesterase compensates for the reduced catalytic activity caused by resistance-conferring mutations in *Tetranychus urticae*. *Insect Biochem Mol Biol.* 2012;42: 212-219.
 34. Shafferman A, Velan B, Ordentlich A, Kronman C, Grosfeld H, Leitner M et al. Substrate inhibition of acetylcholinesterase: residues affecting signal transduction from the surface to the catalytic center. *EMBO J.* 1992;11: 3561-3568.
 35. Boublik Y, Saint-Aguet P, Lougarre A, Arnaud M, Villatte F, Estrada-Mondaca S et al. (2002) Acetylcholinesterase engineering for detection of insecticide residues. *Protein Eng.* 2002;15: 43-50.

Table 1. History and phenotypic classification of salmon lice samples included in the study

The eight salmon lice strains included in the current study are presented with their treatment history with azamethiphos prior to parasite collection (Ls A, Ls G, Ls B, Ls H, Ls V and Ls F) or in the laboratory (Ls H-s and Ls 1998). The salmon lice strains were exposed to azamethiphos for 60 minutes and/or 24 hours in biological assays (bioassays) to detect their sensitivity to the chemical. This information is stated in the table. Whether or not salmon infested with salmon lice from the different strains were subjected to small scale treatment trials to detect treatment efficacies are also given in the table. The small scale treatment of salmon infested with Ls F was performed to detect which genotypes of the parasite that died at different time points during and after the treatment.

Strain	History	Lice bioassay	Small scale fish treatment
Ls A	Sampled in 2010, cultivated for 10 generations, never treated with azamethiphos	60 min. 24 h.	+
Ls G	Sampled in 2004, cultivated for 15 generations, not treated with azamethiphos for 8 years prior to sampling	60 min. 24 h.	-
Ls B	Sampled in 2008, site treated 3 times with azamethiphos the last two years prior to sampling	60 min.	-
Ls H	Sampled in 2011, site treated with azamethiphos more than 5 times for the last two years	60 min. 24 h.	+
Ls H-s	Surviving parasites after a small-scale lab-treatment of Ls H with azamethiphos	-	-
Ls V	Sampled in 2012 immediately after an azamethiphos treatment. The site had been treated with azamethiphos more than 5 times for the last two years	60 min.*	-
Ls F	Sampled in 2013, site treated with azamethiphos more than 5 times for the last two years	24 h.	+**
Ls 1998	Surviving parasites after an azamethiphos bioassay selection experiment in 1998, stored at -80 °C until analysis	-	-

* Tested at one concentration only

** Performed to identify when the different genotypes detached

Table 2: The results from bioassays with 60 minutes and 24 hours exposure to azamethiphos

The results from bioassays with 60 minutes and 24 hours exposure to azamethiphos are given as EC₅₀-values (the concentration that immobilizes 50 % of the parasites) in µgL⁻¹ with 95 % confidence intervals (CI). The bioassay results were modelled using probit modelling in JMP 10 (SAS Institute Inc., Cary, NC, USA). The results from the small scale treatments of salmon infested with Ls A and Ls H in a 30 minute bath treatment with 0.1 mgL⁻¹ azamethiphos and an untreated control group. The results from the small scale treatments were calculated using bootstrapping and are given as percent effect with 95 % CI.

Sea lice strain	EC ₅₀ , 60-min bioassay	EC ₅₀ , 24-h bioassay	Percent efficacy, small scale treatment	Classification
Ls A	2.1 (1.3-3.5)*	0.12 (0.11-0.14)*	100 (90-100)* 98 (82-100)	Sensitive
Ls G	1.8 (1.4-2.5)	0.16 (0.10-0.27)		Sensitive
Ls B	4.5 (1.9-10.7)	-	-	Reduced sensitivity
Ls H	60 (17-216)*	2.1 (1.5-2.7)*	50 (39-61)*	Resistant
Ls V	>50**	-	-	Resistant
Ls F	-	3.3 (1.9-5.6)	-	Resistant

*Remodeled from data presented in Helgesen and Horsberg [10]

**Tested at one concentration only

Table 3: Frequency of the Phe->Tyr change in codon 362 of *ace1a* (*L. salmonis*), corresponding to codon 331 in *T. californica*.

Strain	Sensitivity	Wild type Phe362/Phe362 frequency (SS)	Heterozygote Phe362/362Tyr frequency (RS)	Homozygote 362Tyr/362Tyr frequency (RR)
Ls A	Sensitive	100 %	0 %	0 %
Ls G	Sensitive	96 %	4 %	0 %
Ls B	Reduced sensitivity	72 %	26 %	2 %
Ls H	Resistant	44 %	36 %	20 %
Ls H-s (azamethiphos treatment)	Resistant	0 %	92 %	8 %
Ls V	Resistant	5 %	35 %	60 %

Table 4. Mortality frequency of homozygote sensitive (SS), heterozygote (RS) and homozygote resistant (RR) parasites over eight days following a 30 minute bath treatment with 0.1 mgL⁻¹ azamethiphos

Mortality frequency of homozygote sensitive (SS), heterozygote (RS) and homozygote resistant (RR) parasites over eight days following a 30 minute bath treatment with 0.1 mgL⁻¹ azamethiphos. Three salmon infested with Ls F were treated and all detached salmon lice were removed during the exposure and the following 2.5 hours. Detached parasites were also removed 24 hours later. They are presented in the “dead” column except two parasites which were excluded as they attached to the tank wall after detaching from the fish. The rest of the parasites were picked off the fish 8 days post treatment and these are presented in the “alive” column. All salmon lice were genotyped by PatoGen AS in Ålesund, Norway using a TaqMan assay.

Genotype	Dead	Alive
SS	10	0
RS	11	14
RR	0	16

Figure legends

Figure 1. Nucleotide alignment of *ace1a*

Nucleotide alignment of *L. salmonis ace1a* from sensitive (Ls A) and resistant (Ls H) salmon lice strains. The changes identified are boxed. Of the three nucleotide changes identified, two were silent changes, *Arg80Arg* and *Ser235Ser*, corresponding to *Glu49* and *Ser176* in *T. californica* AChE, respectively. The non-silent T->A change led to the substitution of Phe to Tyr residue at 362 amino acid position corresponding to *Phe331* in *T. californica* AChE.

Figure 2. Nucleotide alignment of *ace1b*

Nucleotide alignment of *L. salmonis ace1b* from sensitive (Ls A) and resistant (Ls H) salmon lice strains. The only change identified in *L. salmonis ace1b* is boxed. This non silent T->C change led to the substitution of Ile to Thr at 433 amino acid position, corresponding to *Ile401* in the *T. californica* AChE.

Figure 3. Amino acid alignment

Alignment of the deduced amino acid sequence of both *L. salmonis ace1a* and *ace1b* in the region where the *Phe362Tyr* change was found, with previously published acetylcholinesterases (AChE) from other insects, arachnida and vertebrates (Insects: *Liposcelis entomophila* Lip_ent, *Bemisia tabaci* Bem_tab, *Blattella germanica* Bla_ger, *Nephotettix cincticeps* Nep_cin, *Ctenocephalides felis* Cte_fel, *Culex pipiens* Cul_pip, *Chilo suppressalis* Chi_sup, *Apis mellifera* Api_mel, *Cimex lectularius* Cim_lec, *Bombyx mandarina* Bom_man, *Bombyx mori* Bom_Mor, *Leptinotarsa decemlineata* Lep_dec, *Drosophila melanogaster* Dros, *Musca domestica* Mus_dom, *Anopheles gambiae* Ano_gam, *Aedes albopictus* Aed_alb, *Culex quinquefasciatus* Cul_qui, Arachnida: *Tetranychus urticae* Tet_urt, *Rhipicephalus decoloratus* Rhi_dec, Vertebrates: *Torpedo californica* Tor_cal, *Homo sapiens* Homosap. *Phe362Tyr* corresponds to *Phe331* in *Torpedo californica* AChE. Phenylalanine at 331 is a highly conserved amino acid in the acetylcholinesterases among all species included. The *Phe331Tyr* change is boxed.

Figure 4. 3D model of important amino acids

Overlay of the predicted three-dimensional positioning of functionally important amino acids in AChE1a wild-type and mutated enzyme from *Lepeophtheirus salmonis*. The changed amino acid (*362Tyr*, corresponding to codon 331 in *T. californica*) is displayed in white. The other amino acids, *Trp115*, *Tyr152*, *Ser230* and *His472* (*Trp84*, *Tyr130*, *Ser200* and *His440* in *T. californica*) are displayed in grey. Other amino acids are not displayed. The *Phe362Tyr* mutation alters the structure and the polarity of the enzymatic pocket. According to the ligand docking model, azamethiphos (green in the wild-type AChE1a, red in the mutated AChE1a) binds differently in the pocket, with H-bonds to both *Tyr152* and *362Tyr* in the mutated enzyme. No H-bonds were predicted between azamethiphos and these two amino acids in the wild-type enzyme. SWISS MODEL in the automated mode [13] (<http://swissmodel.expasy.org/>) was used for modelling of the protein, the molecular docking server (<http://www.dockingserver.com/web>) was used to dock azamethiphos to the protein, and Chimera 1.10.1. (<http://www.cgl.ucsf.edu/chimera/>) was used to illustrate the positions.

Figure 5. High resolution melt plots

High Resolution Melt (HRM) Analysis separated the samples with and without the *Phe362Tyr* mutation (numbering from *L. salmonis*). HRM was based on differences in the shapes of their melt curve that reflects the differences in their genotypes. The Green cluster represents samples homozygous (RR) for the *Phe362Tyr* mutation, Blue cluster represents samples heterozygous (RS) for the *Phe362Tyr* mutation and Red cluster represents the wild type (SS) samples without the *Phe362Tyr* mutation, respectively. All the three clusters were clearly separated from each other on the HRM plot.

Figure 6. Survival analysis plot

Kaplan-Meier survival plot of all three genotypes: homozygote sensitive (SS), heterozygote (RS) and homozygote resistant (RR). Three salmon infested with Ls F were treated for 30 minutes with 0.1 mgL^{-1} azamethiphos in a bath treatment. All detached salmon lice were removed during the exposure and the following 2.5 hours. Detached parasites were also removed 24 hours later. Two parasites were excluded

from the analysis as they attached to the tank wall after detaching from the fish. The rest of the parasites were picked off the fish 8 days post treatment. These salmon lice were regarded as alive, while the detached were regarded as dead. All salmon lice were genotyped by PatoGen AS in Ålesund, Norway using a TaqMan assay. The upper dotted line is the RR group, the solid line is the RS group, while the lower broken line is the SS group. One of the RS parasites died between 200 minutes and 24 hours after start of exposure, but the exact time is unknown. In this plot the time of death is set to 250 minutes. The cut-off limit is set to 300 minutes.

Figure 7. Residual enzyme activity after *in vitro* inhibition

The relative residual AChE activity with or without propoxur in susceptible (SS, n=20) and resistant (RR, n=21) lice is displayed. A statistically significant effect on residual AChE activity was found between the two groups, indicating a protective effect of the *Phe362Tyr* mutation against propoxur inhibition in homogenates ($p < 0.0001$, ANOVA). The box plots indicate the group median, 75% and 25% quantiles, and whiskers (JMP, SAS Institute). Different letters indicate a statistically significant difference.

Figure 8. Residual enzyme activity after *in vivo* inhibition

AChE activity (mU/mg protein) in susceptible (SS) and resistant (RR) lice after treatment with $0 \mu\text{gL}^{-1}$ (control) or $2 \mu\text{gL}^{-1}$ azamethiphos for 24 hours. No difference was observed between the control groups. A statistically significant decrease in the residual activity after $2 \mu\text{gL}^{-1}$ azamethiphos treatment was found in both strains ($p < 0.0001$, Steel-Dwass method). In addition, there was a statistically significant difference between the two treated groups (SS- $2 \mu\text{gL}^{-1}$ and RR- $2 \mu\text{gL}^{-1}$; $p = 0.019$, Steel-Dwass method) indicating a protective effect of the *Phe362Tyr* mutation against azamethiphos bath treatment). The box plots indicate the group median, 75% and 25% quantiles, and whiskers (JMP, SAS institute). Different letters indicate a statistically significant difference.

Supporting Information captions

S1 file. PDB-file of the wild-type AChE1a in *Lepeophtheirus salmonis*

S2 file. PDB-file of the mutated AChE1a in *Lepeophtheirus salmonis*

S1 Table. Frequency of the *Ile433Thr* change in AChE1b in *Lepeophtheirus salmonis*

Figure 1. Nucleotide alignment of *ace1a*

Ls A	1	ATGTGGATTCAAGTCAGAAAACAAAACCTTTGGTCTTTACTTTAATAAGATATTAGTGTAT	60
Ls H		ATGTGGATTCAAGTCAGAAAACAAAACCTTTGGTCTTTACTTTAATAAGATATTAGTGTAT	
Ls A	61	TTGCTCACTTTTGCATGGAGCGTGGGTGCTATCGTACAAGACAATTTGGTGATCACCACA	120
Ls H		TTGCTCACTTTTGCATGGAGCGTGGGTGCTATCGTACAAGACAATTTGGTGATCACCACA	
Ls A	121	AAAAAAGGAAAGATCCGAGGTGTTACTCTTAAATCTGCAACAAATAGGGAAGTAGATGCA	180
Ls H		AAAAAAGGAAAGATCCGAGGTGTTACTCTTAAATCTGCAACAAATAGGGAAGTAGATGCA	
Ls A	181	TGGTATGGGATTCATACGCACAACCTCCAGTGGGTAATCTTCGATTTTCGTCACCCTAGA	240
Ls H		TGGTATGGGATTCATACGCACAACCTCCAGTGGGTAATCTTCGATTTTCGTCACCCTAGG	
Ls A	241	CACATTGATGCCTGGGAGGGGATTAAGAAACGACCCGACCTCCAAATTCTTGTATTCAA	300
Ls H		CACATTGATGCCTGGGAGGGGATTAAGAAACGACCCGACCTCCAAATTCTTGTATTCAA	
Ls A	301	GTAGTTGATACATTATTTCCAGGCTTTGAAGGCGCAGAAATGTGGAATGTGAATACAGAG	360
Ls H		GTAGTTGATACATTATTTCCAGGCTTTGAAGGCGCAGAAATGTGGAATGTGAATACAGAG	
Ls A	361	CCCAGCGAGGACTGTCTTTATTTAAGTGTCCATGTCCCTAAACCCCGTCTACAGGATCA	420
Ls H		CCCAGCGAGGACTGTCTTTATTTAAGTGTCCATGTCCCTAAACCCCGTCTACAGGATCA	
Ls A	421	GCTGTTCTGGTATGGATCTACGGTGGAGGATTTTATTCTGGAACCTTACTCTGGAAGTC	480
Ls H		GCTGTTCTGGTATGGATCTACGGTGGAGGATTTTATTCTGGAACCTTACTCTGGAAGTC	
Ls A	481	TATGATCCACGAGTCTTGTGTGCAAGAAAACATAATCTTCGTTGCCATGCAATATCGT	540
Ls H		TATGATCCACGAGTCTTGTGTGCAAGAAAACATAATCTTCGTTGCCATGCAATATCGT	
Ls A	541	GTTGCAAGTTTAGGGTTCCTTTTCTTTGATACGGAGGATGTTCCCTGGAAATGCGGGATTG	600
Ls H		GTTGCAAGTTTAGGGTTCCTTTTCTTTGATACGGAGGATGTTCCCTGGAAATGCGGGATTG	
Ls A	661	TATGATCAAATGATGGCTCTCCAATGGGTAAGAAACAATATAGAGGAATTTGGTGGTGAT	720
Ls H		TATGATCAAATGATGGCTCTCCAATGGGTAAGAAACAATATAGAGGAATTTGGTGGTGAT	
Ls A	721	CCTGATAAAATCACCATTTTCGGAGAGTCCGCCGGTGGTTGCTCCGTAGCCCTTCACCTC	780
Ls H		CCTGATAAAATCACCATTTTCGGAGAGTCCGCCGGTGGTTGCTCCGTAGCCCTTCACCTC	
Ls A	781	CTTTCCCCTCTCAAGGAACCTGTTTTCTCAAGCCATCATGCAGAGTGCTTCAGCTCTT	840
Ls H		CTTTCCCCTCTCAAGGAACCTGTTTTCTCAAGCCATCATGCAGAGTGCTTCAGCTCTT	
Ls A	841	GTTCCATGGGGAGTCATCACAAAAAAGAAAGTATTATTCGTGGTCCGAGGCTTGCGGAA	900
Ls H		GTTCCATGGGGAGTCATCACAAAAAAGAAAGTATTATTCGTGGTCCGAGGCTTGCGGAA	
Ls A	901	ATGATGAGCTGTCCCTACGATGAAAAAATACAAAGGCCATGATTGAATGCCTGCGGCAA	960
Ls H		ATGATGAGCTGTCCCTACGATGAAAAAATACAAAGGCCATGATTGAATGCCTGCGGCAA	
Ls A	961	AAGGATGCAACAGAGATGGTAAATCAGGAGTGGATTGGTATCATTCTGGGATTGCAGAG	1020
Ls H		AAGGATGCAACAGAGATGGTAAATCAGGAGTGGATTGGTATCATTCTGGGATTGCAGAG	
Ls A	1021	TTCCCTTTTGTTCCTATTGTGGATGGAAGTTTCTTGGATGAGAGCCCTGGAAAAATCTCTT	1080
Ls H		TTCCCTTTTGTTCCTATTGTGGATGGAAGTTTCTTGGATGAGAGCCCTGGAAAAATCTCTT	
Ls A	1081	ACAACAAAGAACTATAAAAAACCAACATTTTAATTGGAGCGAATAAGGAAGAAGGGAAT	1140
Ls H		ACAACAAAGAACTATAAAAAACCAACATTTTAATTGGAGCGAATAAGGAAGAAGGGAAT	
Ls A	1141	TATTACATCATGTACTATCTTACAGATCTCTTAAAAATACGGAGAGCGTCTATGTTGAT	1200
Ls H		TATTACATCATGTACTATCTTACAGATCTCTTAAAAATACGGAGAGCGTCTATGTTGAT	

Arg80Arg

Ser235Ser

Phe362Tyr

Ls A 1201 CGAACAGATTTTCATTCGAAGTGTTTCAGAATTGAACCACTATGTAAAAAATGGGAAGA 1260
 Ls H CGAACAGATTTTCATTCGAAGTGTTTCAGAATTGAACCACTATGTAAAAAATGGGAAGA

Ls A 1261 GAGGCAATAACTTTTGGAGTACACAGATTGGCTCAATCCAAACGATCCCATAAAAAATAGA 1320
 Ls H GAGGCAATAACTTTTGGAGTACACAGATTGGCTCAATCCAAACGATCCCATAAAAAATAGA

Ls A 1321 GAAGCAATTGATCGCATGGTCGGTGACTATCAATTCATTTGCCCACTGCTGACTTTGCT 1380
 Ls H GAAGCAATTGATCGCATGGTCGGTGACTATCAATTCATTTGCCCACTGCTGACTTTGCT

Ls A 1381 CGTATTTATGCTAGTACAGGAAATAATGTATACATGTACTATTTCACTGAGCGATCTTCC 1440
 Ls H CGTATTTATGCTAGTACAGGAAATAATGTATACATGTACTATTTCACTGAGCGATCTTCC

Ls A 1441 ACTAGCCCATGGCCAACATGGTCTGGTGTACTTCATGGCGATGAAATTGCTTTTTGTTTTT 1500
 Ls H ACTAGCCCATGGCCAACATGGTCTGGTGTACTTCATGGCGATGAAATTGCTTTTTGTTTTT

Ls A 1501 GGAGAGGCCCTTAATAAGTCAAAGAATTATGATAAGTCAGAAATTGCCCTATCAAAAAGA 1550
 Ls H GGAGAGGCCCTTAATAAGTCAAAGAATTATGATAAGTCAGAAATTGCCCTATCAAAAAGA

Ls A 1551 ATGATGGGATATTGGGCTAATTTTGTCTAAAACGGGAATCCGAGTCTTCTGCTGATGGA 1610
 Ls H ATGATGGGATATTGGGCTAATTTTGTCTAAAACGGGAATCCGAGTCTTCTGCTGATGGA

Ls A 1611 ACTTGGAGCACAAATTATTGGCCTTTACATACGCCGACAAAACAGGAGGTACTTGAATTA 1670
 Ls H ACTTGGAGCACAAATTATTGGCCTTTACATACGCCGACAAAACAGGAGGTACTTGAATTA

Ls A 1671 AACGCCAATTATTCTCGAGTTCTCGAGGGTCTTCGAGTTAAAAAATGTGCCTTTTGGAAA 1730
 Ls H AACGCCAATTATTCTCGAGTTCTCGAGGGTCTTCGAGTTAAAAAATGTGCCTTTTGGAAA

Ls A 1731 AAATATCTACCTAACTTTTATCATTAACCTCAAACAATGGAGACCATCAGTGTCAAACA 1790
 Ls H AAATATCTACCTAACTTTTATCATTAACCTCAAACAATGGAGACCATCAGTGTCAAACA

Ls A 1791 TCCTCGACATCATCTCCCTCATGCTGTAAAGATGGAACATGCTGCAACAGTGGGACGTCC 1850
 Ls H TCCTCGACATCATCTCCCTCATGCTGTAAAGATGGAACATGCTGCAACAGTGGGACGTCC

Ls A 1851 TTTGCATCCACGTTCTTTTCAGTATTCATTGTAACAACGTCGGTGCATCTATTTTATAAT 1910
 Ls H TTTGCATCCACGTTCTTTTCAGTATTCATTGTAACAACGTCGGTGCATCTATTTTATAAT

Ls A 1911 TTTAAATTTAGTGTAATTTACTCAAATATTAATAATGGAAAACGTGTGA 1970
 Ls H TTTAAATTTAGTGTAATTTACTCAAATATTAATAATGGAAAACGTGTGA

Figure 2. Nucleotide alignment of *ace1b*

Ls A	1	ATGTGGATTCAAGTCCGAAAAACAACCTTAGGTCTTTCCTTTGAAAGGATATTAGTGTAT	60
Ls H		ATGTGGATTCAAGTCCGAAAAACAACCTTAGGTCTTTCCTTTGAAAGGATATTAGTGTAT	
Ls A	61	TTACTCACATTGTCATGGAGTCTGGGATCCATCGTACAAGAAGATTTGGTGATCACCACA	120
Ls H		TTACTCACATTGTCATGGAGTCTGGGATCCATCGTACAAGAAGATTTGGTGATCACCACA	
Ls A	121	AGAAAAGGAAAGATCCGAGGTGTTACTCTGAAATCTGCAACAAATAAGGAAGTAGATGCA	180
Ls H		AGAAAAGGAAAGATCCGAGGTGTTACTCTGAAATCTGCAACAAATAAGGAAGTAGATGCA	
Ls A	181	TGGTATGGGATACCATACGCACAACCTCCCCTGGGTAATCTTCGATTTTCGTACCCCCAAA	240
Ls H		TGGTATGGGATACCATACGCACAACCTCCCCTGGGTAATCTTCGATTTTCGTACCCCCAAA	
Ls A	241	GACATTAATGCCTGGGATGGGATGAAAGAAACGACCAAACATCCAAATTCTTGATTCAA	300
Ls H		GACATTAATGCCTGGGATGGGATGAAAGAAACGACCAAACATCCAAATTCTTGATTCAA	
Ls A	301	GTAGTTGATACATTTTTTCCGGGCTTTGAAGGCTCAGAGATGTGGAATACAAATACTGAG	360
Ls H		GTAGTTGATACATTTTTTCCGGGCTTTGAAGGCTCAGAGATGTGGAATACAAATACTGAG	
Ls A	361	CAAAGCGAGGACTGCCTTTACTTAAGTGTTTCATGCCCCTAAACCCCGTCCTACAAAATCA	420
Ls H		CAAAGCGAGGACTGCCTTTACTTAAGTGTTTCATGCCCCTAAACCCCGTCCTACAAAATCA	
Ls A	421	GCTGTTCTGGTATGGATCTACGGTGGAGGATTTTATTCTGGAACCTCAACTCTGGAACTC	480
Ls H		GCTGTTCTGGTATGGATCTACGGTGGAGGATTTTATTCTGGAACCTCAACTCTGGAACTC	
Ls A	481	TATGATCCACGAGTTCCTGTGTGAGAAGAAAACATAATCTTCGTGGCATAACAATATCGT	540
Ls H		TATGATCCACGAGTTCCTGTGTGAGAAGAAAACATAATCTTCGTGGCATAACAATATCGT	
Ls A	541	GTTGCAAGTTTAGGATTCCTATTCTTTGATACGGAGGATGTTCCCTGGAAATGCGGGATTG	600
Ls H		GTTGCAAGTTTAGGATTCCTATTCTTTGATACGGAGGATGTTCCCTGGAAATGCGGGATTG	
Ls A	601	TATGATCAAATGATGGCTCTCCAATGGGTAAAGAACAATATAGAGGCATTTGGTGGTGAT	660
Ls H		TATGATCAAATGATGGCTCTCCAATGGGTAAAGAACAATATAGAGGCATTTGGTGGTGAT	
Ls A	661	CCTGATAAAATCACCATTTTTGGAGAGTCCGCCGGTGGTTGCTCCGTAGCCCTTCATCTC	720
Ls H		CCTGATAAAATCACCATTTTTGGAGAGTCCGCCGGTGGTTGCTCCGTAGCCCTTCATCTC	
Ls A	721	CTTTCTCCACTCTCAAGGAACCTATTCTCTCAAGCCATTATGCAAAGTTCTTCAGCTCTT	780
Ls H		CTTTCTCCACTCTCAAGGAACCTATTCTCTCAAGCCATTATGCAAAGTTCTTCAGCTCTT	
Ls A	781	GTTCCATGGGGAGTCATATCAAAAAAAGAAAGTATCCGTCGTGGTGAAGACTTGCAGAA	840
Ls H		GTTCCATGGGGAGTCATATCAAAAAAAGAAAGTATCCGTCGTGGTGAAGACTTGCAGAA	
Ls A	841	GAGATGCGTTGTCCTTATGGTGAAAAATAATACCAATGCTATGATTGAATGCCTGCTGCAA	900
Ls H		GAGATGCGTTGTCCTTATGGTGAAAAATAATACCAATGCTATGATTGAATGCCTGCTGCAA	

Ls A 901 AAGGACGCAACAGAGTTGGTCAACCAGGAGTGGAGTGGTACCGTCTTTGGGATTTTCAGAG 960
Ls H AAGGACGCAACAGAGTTGGTCAACCAGGAGTGGAGTGGTACCGTCTTTGGGATTTTCAGAG

Ls A 961 TTCCCATTTGTTCCAATTGTGGATGGAAAATTCATGGATAAAACCCCTGAAAAATCTCTT 1020
Ls H TTCCCATTTGTTCCAATTGTGGATGGAAAATTCATGGATAAAACCCCTGAAAAATCTCTT

Ls A 1021 AAAGAAAAGGACTATAAAAAAACCAACATTTTAAATGGGAGTCAATAAGGACGAAGGGAAC 1080
Ls H AAAGAAAAGGACTATAAAAAAACCAACATTTTAAATGGGAGTCAATAAGGACGAAGGGAAC

Ls A 1081 TTTTTCATCATGTATTATCTTCCAGAACTCTTCAAAAAAACGAAAACGTTTATATTAAC 1140
Ls H TTTTTCATCATGTATTATCTTCCAGAACTCTTCAAAAAAACGAAAACGTTTATATTAAC

Ls A 1141 CGAACAGATTTTCATCCGCAGTGTTCAGATTTGAACATCTATGTGAACAATGCAGGAAGA 1200
Ls H CGAACAGATTTTCATCCGCAGTGTTCAGATTTGAACATCTATGTGAACAATGCAGGAAGA

Ls A 1201 GAGGCAATAACTTTTGAATACACAGATTGGCTCAATCCAAACGATCCCATAAAAAATAGA 1260
Ls H GAGGCAATAACTTTTGAATACACAGATTGGCTCAATCCAAACGATCCCATAAAAAATAGA

Ile433Thr

Ls A 1261 GAAGCAATTGATCGCATGGTCGGTACTATCAATTCATTTGCCCAACTGCTGACTTTGCT 1320
Ls H GAAGCAATTGATCGCATGGTCGGTACTATCAATTCATTTGCCCAACTGCTGACTTTGCT

Ls A 1321 CGTATTTATGCTAGTACAGGAAATAATATATACATGTACTATTTCACTGAGCGATCTTCC 1380
Ls H CGTATTTATGCTAGTACAGGAAATAATATATACATGTACTATTTCACTGAGCGATCTTCC

Ls A 1381 ACTAGCCCATGGCCAACATGGTCTGGTGTACTTCATGGCGATGAAATTGCTTTTGTTTTT 1440
Ls H ACTAGCCCATGGCCAACATGGTCTGGTGTACTTCATGGCGATGAAATTGCTTTTGTTTTT

Ls A 1441 GGAGAGCCCCTAAATACGTCAAAAAATTATGATGATTGAGAAATGCCTTATCAAAAAGA 1500
Ls H GGAGAGCCCCTAAATACGTCAAAAAATTATGATGATTGAGAAATGCCTTATCAAAAAGA

Ls A 1501 ATAATGAGCTATTGGGCTAATTTTGCAAAAACGGGAACCCGAATGTTTGGCTAATGGA 1550
Ls H ATAATGAGCTATTGGGCTAATTTTGCAAAAACGGGAACCCGAATGTTTGGCTAATGGA

Ls A 1551 AACTACAGCAACAAAATCTGGCCCTTACATACACCAATAAAACAGGAGGTACTTGAACTA 1610
Ls H AACTACAGCAACAAAATCTGGCCCTTACATACACCAATAAAACAGGAGGTACTTGAACTA

Ls A 1611 AATGCAAATTATTCGAGTTTTTGAGGGGCTTCGAGTTAGAAAATGTGCTTTTTGGAAA 1670
Ls H AATGCAAATTATTCGAGTTTTTGAGGGGCTTCGAGTTAGAAAATGTGCTTTTTGGAAA

Ls A 1671 ACATATCTTCCCTAAGCTTTTATCATTAACCTCAAACAATACAAAGTCTGAAGTTGTAACC 1730
Ls H ACATATCTTCCCTAAGCTTTTATCATTAACCTCAAACAATACAAAGTCTGAAGTTGTAACC

Ls A 1731 AATCCGTCATAA 1742
Ls H AATCCGTCATAA

Figure 3. Amino acid alignment



Figure 4. 3D model of important amino acids

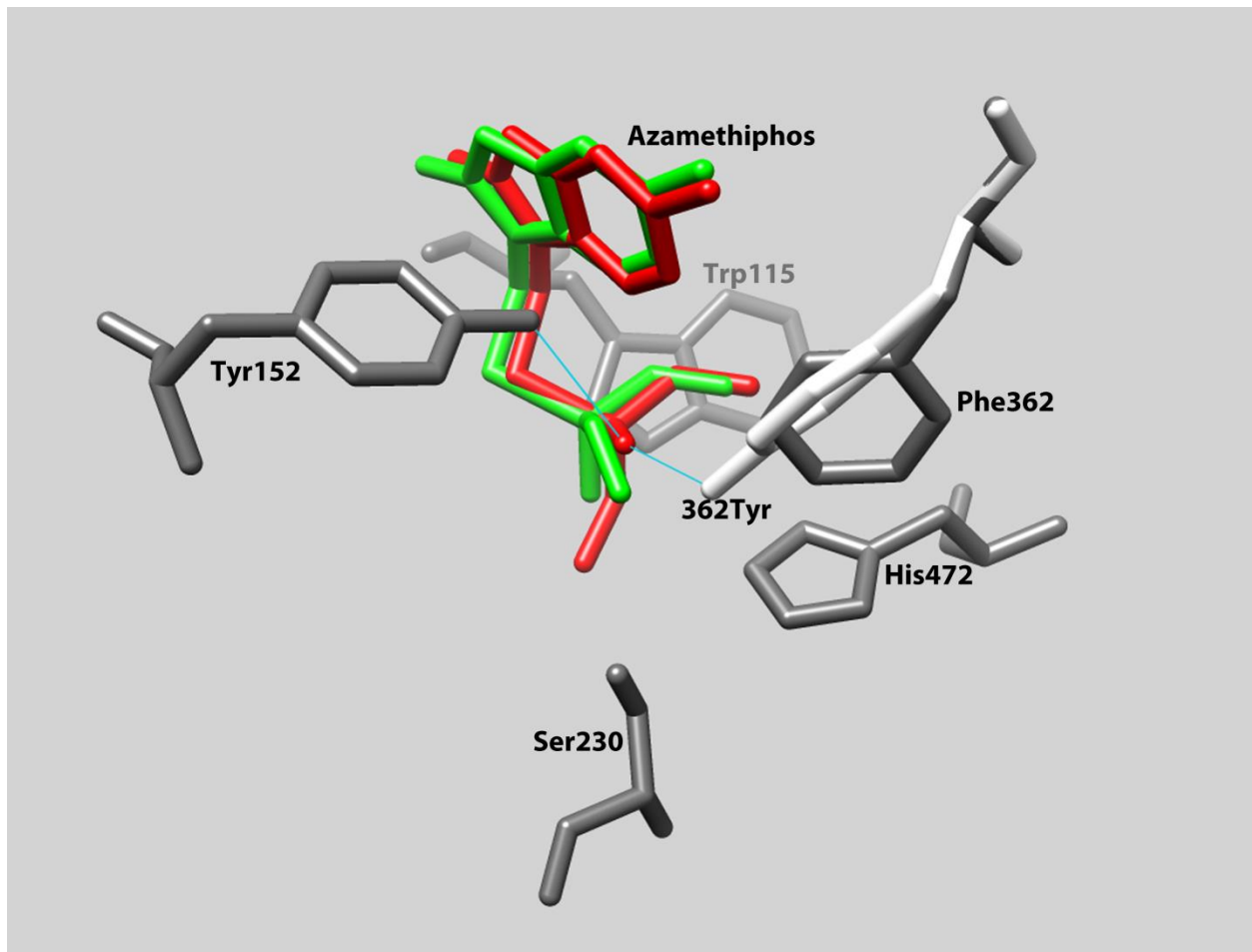


Figure 5. High resolution melt plots

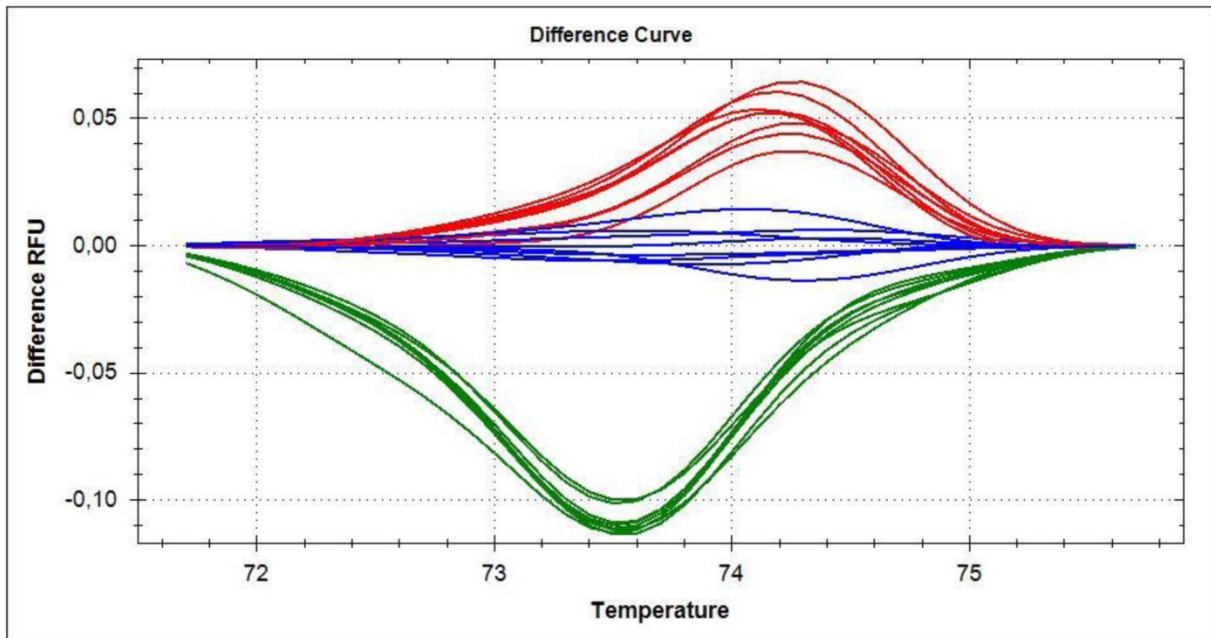


Figure 6. Survival analysis plot

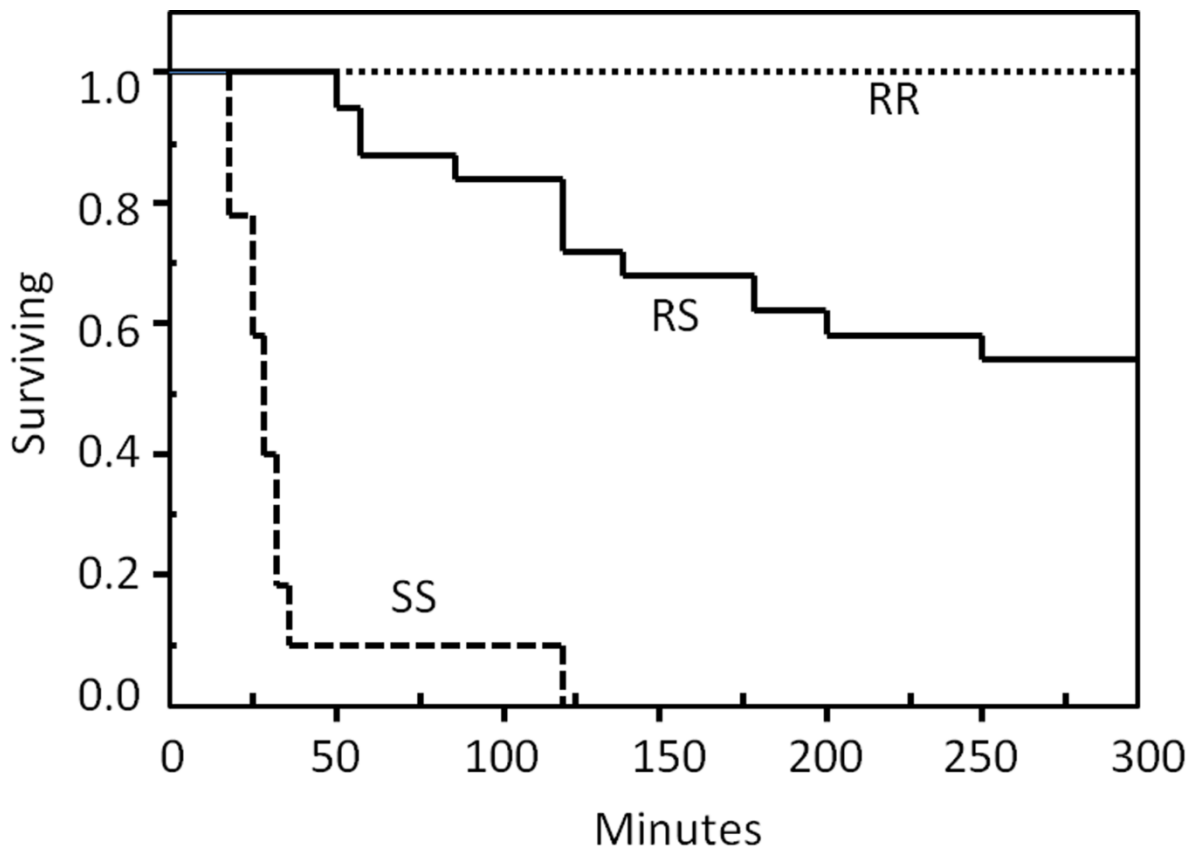


Figure 7. Residual enzyme activity after *in vitro* inhibition

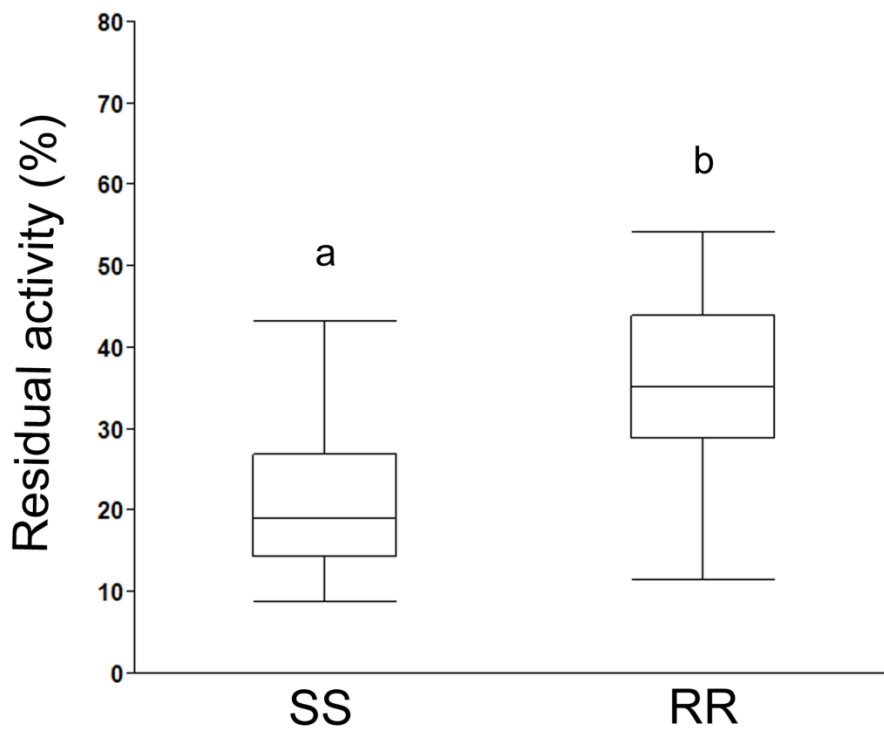
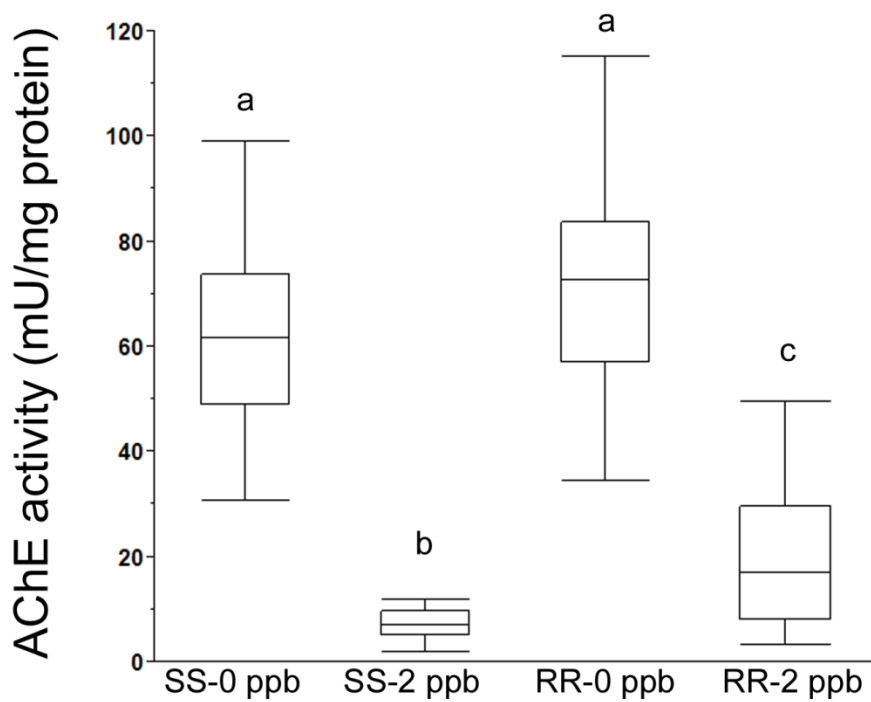


Figure 8. Residual enzyme activity after *in vivo* inhibition



IV

Voltage gated sodium channel genes in the salmon louse (*Lepeophtheirus salmonis*): Gene characterizations and sequence analysis of knockdown resistance-homologous regions in pyrethroid resistant parasites

Kari Olli Helgesen^{a*}, Frank Nilsen^b, Heidi Kongshaug^b, Kiranpreet Kaur^a and Tor Einar Horsberg^a

^aNorwegian University of Life Sciences, School of Veterinary Science, Sea Lice Research Centre, Pb. 8146 Dep., 0033 Oslo, Norway

^bUniversity of Bergen, Sea Lice Research Centre, Pb. 7803, 5020 Bergen, Norway

*Corresponding Author. E-mail: kari.helgesen@nmbu.no, phone number: +47 22597090

Abstract

The salmon louse *Lepeophtheirus salmonis* is a prominent concern for all salmonid producing countries in the Northern Hemisphere. Most fish farmers are dependent on chemical treatments in order to control the number of parasites. Pyrethroids are one of the few available treatment options, however recurrent treatments have resulted in pyrethroid resistance in *L. salmonis*. Despite the fact that the mechanisms for pyrethroid resistance have been found in several other arthropod species, it is as yet mostly unknown in salmon lice. One of the most commonly observed pyrethroid resistance mechanisms in arthropods is target-site mutations in the voltage gated sodium permeable channel (Na_v) gene. The aim of the current study was to isolate the Na_v genes in *L. salmonis*, in order to screen for mutations in these genes in pyrethroid resistant parasites from different parts of Norway. The gene $\text{Na}_v1.1$ was fully sequenced using RACE PCR, while $\text{Na}_v1.2$ and $\text{Na}_v1.3$ was identified through a homology search in the salmon louse genome. A quantitative PCR study of these three genes was performed on resistant and sensitive salmon lice, which identified $\text{Na}_v1.1$ as the most expressed gene. Homologous sequences to recognised mutation hot spots were identified in $\text{Na}_v1.1$. PCR and direct sequencing were employed to investigate 46 salmon lice from four different areas of Norway, all survivors of deltamethrin laboratory treatments, for mutations in these hot spots. Compared to the sequences collected from a fully sensitive strain of salmon lice, no mutations were identified. However, three synonymous single nucleotide polymorphisms (SNP) were detected in the sequences included in the study. A large amount of heterogeneity in these SNPs was seen among the studied salmon lice. A combination of these two findings suggest that target-site mutation in $\text{Na}_v1.1$ is not the mechanism behind pyrethroid resistance in salmon lice in Norway and that future research on resistance mechanisms should be focused elsewhere.

Keywords:

Salmon lice, *Lepeophtheirus salmonis*, resistance, pyrethroids, sodium channel, mutations

1. Introduction

Infestations with the salmon (or sea) louse *Lepeophtheirus salmonis* are a constant challenge to both the fish and the fish farmers in salmonid aquaculture in the Northern Hemisphere (Jones and Beamish, 2011). Control of this parasite is mainly dependent on chemical treatments, including pyrethroids. Pyrethroids belong to a class of chemicals which are used for bath treatments against all parasitic instars of *L. salmonis* (Hart et al., 1997). In the year 2000 the use of pyrethroids against salmon lice was reported in Scotland, the Faroe Islands, Ireland, the United States and Norway (Roth, 2000). In Norway pyrethroids were introduced to the market in 1996 (Grave et al., 2004).

In 1998, just a couple of years following the introduction of pyrethroids to the Norwegian market, the first accounts of reduced treatment efficacy were reported. Reduced sensitivity in the parasites was confirmed by laboratory assays (Sevatdal and Horsberg, 2000). Reports of reduced treatment efficacy, due to reduced sensitivity, also exist from Scotland and Canada (Sevatdal and Horsberg, 2003; Sevatdal et al., 2005a; Whyte et al., 2014). Chilean salmonid aquaculture faces challenges with the sea louse *Caligus rogercresseyi*. In Chile pyrethroids have been extensively used for treatments of sea lice and resistance has thus developed (Bravo et al., 2013; Helgesen et al., 2014). In spite of resistance issues, due to the limited availability of alternative chemical agents for treatments of salmon lice, pyrethroids are still regarded as important treatment agents (Torrissen et al., 2013).

In arthropods, pyrethroids' target protein is the voltage gated sodium permeable channel (Na_v) in nerve cell membranes. It is homologous to the α -subunit of vertebrate sodium channels (Bloomquist, 1996; Barzilai et al., 2012). The Na_v genes contain four internally homologous regions; domain I to IV, each containing six transmembrane segments. Pyrethroids alter both the activation and the inactivation of the channels which lead to discontinuity of the normal neural impulse, subsequently paralysis and eventual death of the exposed arthropod (Bloomquist, 1996).

In arthropods acting as agricultural pests, different pyrethroid resistance mechanisms have been discovered. These include increased metabolism or detoxification of the chemicals, including increased expression of carboxylesterases, cytochrome P450s, glutathione-S-transferases and superoxide dismutases (Vontas et al., 2001; Müller et al., 2007; Ranson et al., 2011; Xu et al., 2013). Furthermore, target-site mutations and various polymorphisms in the Na_v gene have also been described. Mutations have been found in all four domains of the gene in pyrethroid resistant arthropods (Wang et al., 2002; Davies et al., 2007; Van Leeuwen et al., 2010) and electrophysiological evidence suggests that a number of these mutations alter the sodium channel in a manner which renders them pyrethroid resistant (Vais et al., 2000).

Both target-site mutation in a Na_v gene and indications of monooxygenase mediated pyrethroid detoxification have been described in salmon lice (Fallang et al., 2005; Sevatdal et al., 2005b). However, none of these mechanisms have a proven association to pyrethroid resistance and have therefore not been developed into *in vitro* resistance testing tools. An enhanced understanding of the underlying mechanisms for pyrethroid resistance in *L. salmonis* is therefore urgently required in order to procure more accurate tools for resistance testing than the biological assays in use today (Sevatdal and Horsberg, 2003; Helgesen and Horsberg, 2013). Knowledge of existing resistance mechanisms may also prove useful when developing new treatments against salmon lice.

The focus in the current study was on the voltage gated sodium channels. The aims were to identify and characterize these genes in *L. salmonis*. Furthermore to investigate into explanations for resistance by comparing the expression levels of these genes in pyrethroid sensitive and pyrethroid resistant parasites and subsequently identify possible target-site mutations in the most frequently expressed Na_v gene.

2. Materials and methods

2.1 Gene identification

tBlastn searches with insects Na_v proteins as query was used to identify homologous Na_v genes in an early assembly of the salmon louse genome (<http://sealouse.imr.no/>). Several putative Na_v contigs were identified which were subsequently used to design primers for rapid amplification of cDNA ends (RACE). This was carried out in order to obtain full-length cDNA sequence of the gene. A mixture of adult female and male salmon lice from an inbred strain, Ls1a (see Hamre et al. (2009)), was used for gene identification. Specific primers for the Na_v gene in salmon lice were constructed using VectorNT19 (Informax Inc, USA). The primers were used for polymerase chain reaction (PCR) and the products applied in direct sequencing. The nucleotide sequences were determined by standard dye terminator chemistry using the Big Dye Terminator v.3.1 (Applied Biosystems, USA) in an ABI 3730XL Analyzer (Applied Biosystems, USA). 5' and 3' ends of partial cDNAs were amplified using RACE with sequence specific primers (outlined below) and SMARTer RACE kit (Clontech, USA) as per manufacturer's instructions. RACE PCR was performed under the following conditions: 94 °C for 2 min; 5 cycles of 94 °C for 30 sec, 72 °C for 3 min; 5 cycles of 94 °C for 30 sec, 70 °C for 30 sec, 72 °C for 3 min; 25 cycles of 94 °C for 30 sec, 68 °C for 30 sec, 72 °C for 3 min; 72 °C for 5 min.

Primers used for 5'RACE:

b2: CAACACTGCCTTTGTGACTCCCTGT
b290: CGCTTGCTACAGTTGCTGTTCTCAATAG
b233: TCTGGAGGGAACTCTGCCGCCATTC

Primers used for 3'RACE:

b114: TAGGAGAGAGGCCCTTATGAGACAA

Both 5'RACE and 3'RACE PCR products were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen, USA) followed by PCR directly on the colonies. Amplicons were obtained using TOPO vector specific primers (outlined below) under PCR conditions: 94 °C for 5 min; 25 cycles of 94 °C for 30 sec, 55 °C for 15 sec, 72 °C for 3 min; 72 °C for 5 min. Amplicons were purified with ExoSAP-IT (USB Corporation, USA) as per manufacturer's instructions. The products were used in direct sequencing applying the aforementioned technic.

TOPO vector specific primers used:

M13 Forward: GTAAAACGACGGCCAG
M13 Reverse: CAGGAAACAGCTATGAC

In order to identify putative Na_v genes in the salmon louse genome, the deduced Na_v 1.1 amino acid sequence was used as a template in a search for homology in the database LiceBase (<https://licebase.org/>). The gene sequences of these identified genes were then collected from the salmon louse genome.

2.2 Genomic organization

The complete cDNA sequence for Na_v1.1, obtained after RACE PCR, was blasted against the *L. salmonis* genome in order to determine genomic organization of the gene in *L. salmonis*. This was performed using the Spidey mRNA-to-genomic alignment program (<http://www.ncbi.nlm.nih.gov/spidey/>). The genomic organization of the other identified Na_v genes was determined directly in the salmon louse genome.

2.3 Alignment and phylogeny

The nucleotide sequence of Na_v1.1 and the Na_v-genes identified from the salmon louse genome were aligned, using Clustal Omega version 1.2.0 with default settings on the website of European molecular biology laboratory/European bioinformatics institute (EMBL-EBI) (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) (Sievers et al., 2011). Deduced amino acid sequences from *L. salmonis* Na_v genes were subsequently aligned with amino acid sequences of Na_v-genes from 6 other organisms, collected from GenBank (GB) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>): *Musca domestica* (X96668.1), *Drosophila melanogaster* (NM_001201672.1), *Apis dorsata* (XM_006613002.1), *Liposcelis bostrychophila* (KC699919.1), *Rattus norvegicus* (L39018.1) and *Danio rerio* (XM_005165770.1). The amino acid sequences of the transmembrane segments of the three *L. salmonis* Na_v genes and the Na_v gene of *M. domestica* were separately aligned. The three Na_v genes in *L. salmonis* were further aligned with the published partial cDNA sequence from a Na_v gene in *L. salmonis* (AJ812299.1).

Construction of the phylogenetic tree involved the alignment of the nucleotide sequences from the three Na_v genes from *L. salmonis* with 13 other sodium channel genes collected from GenBank, using the multiple alignment program MUSCLE. The nucleotide sequence from the 6 aforementioned species were included, as well as *Aedes albopictus* (AY663384.1), *Anopheles gambiae* (AM422833.1), *Megachile rotundata* (XM_003704833.1), *Bombus terrestris* (XM_003397716), *Cimex lectularis* (FJ031996.1), *Cancer borealis* (EF089568.2) and *Tetranychus cinnabarinus* (JX290514.1). The tree was constructed using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-32092.1101) is shown. Credibility of the internal branch was assessed using bootstrapping method (1000 bootstrap replicates). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. The topology with superior log likelihood value was subsequently selected. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing

gaps and missing data were eliminated. There were a total of 2765 positions in the final dataset. Evolutionary analyses were conducted in the software MEGA6 (Tamura et al., 2013).

2.4 Samples for qPCR and mutation screening

Salmon lice for sequence analysis were collected from pyrethroid resistant strains from four different counties in Norway and from one sensitive strain from a fifth county. The sensitivity status of the resistant salmon louse strains, Ls Fr, Ls V, Ls D and Ls Fu, was based on field reports of treatment efficacy and bioassay results. The sensitive strain Ls A was an in-house lab strain originally collected from an area without pyrethroid treatment history. This strain was investigated for pyrethroid sensitivity by small-scale laboratory treatment trials and bioassays, as described in Helgesen and Horsberg (2013).

The experiments were conducted at the Norwegian Institute for Water Research (NIVA)'s marine research station at Drøbak, Norway. Atlantic salmon (*Salmo salar*), used in the experiments, weighed between 100 and 500 grams and originated from the commercial supplier Sørsmolt in Kragerø, Norway. Prior to all handling procedures, the fish were anesthetized, using Finquel vet (Tricain mesilat, Western Chemical Inc., USA) dissolved in fresh water giving a final concentration of 125 mg/l sea water. The fish were kept under constant observation during anaesthesia and deltamethrin treatment.

Ls Fr came from the southern part of Mid-Norway and was collected at a fish processing plant. The lice were transported to NIVA overnight by post in cooled sea water and 45 salmon lice were manually placed on 17 fish kept in a 1000 litre fibre-glass tank supplied with running sea water (salinity 32 ‰, temperature: 8 °C). Six days after salmon lice infestation, these fish were treated with a concentration of 0.002 mg deltamethrin/l sea water using ALPHA MAX (deltamethrin 10 mg/ml, Pharmaq AS, Norway). The duration of the treatment was 30 minutes. The applied concentration was achieved by mixing the correct amount of ALPHA MAX with one litre sea water and subsequently adding the solution to the fish tank while stirring vigorously. The water inlet and outlet were closed during the treatment period; however aeration of the sea water continued through the entire procedure. After 30 minutes, water was drained from the tank until the dorsal fins of the fish were above water level. The tank was subsequently filled with fresh sea water and normal water circulation was re-established. Eight days after treatment all living parasites (6 adult females and 1 adult male) were collected from the fish.

Ls V originated from the northern part of Mid-Norway, and was collected at a processing plant. A total of 80 adult female lice were placed on fish at NIVA, eight salmon lice on each of the respective ten fish held in individual 40-litre fish glass tanks. The fish were subjected to deltamethrin treatment four days following infestation, using the same treatment protocol described in the previous paragraph. Fresh deltamethrin working solution was prepared for each fish tank. Seven days after treatment all living salmon lice (34 lice) were collected from the fish.

Ls D originated from the southern part of Northern Norway and arrived at NIVA one day after they were collected at a fish farm. 40 adult female salmon lice were divided amongst eight fish which were subsequently placed in individual tanks. The same treatment protocol as for Ls V was applied four days later. All surviving lice (21 lice) were collected seven days after treatment.

Ls Fu came from the Hardanger area in the south-western part of Norway. Eggstrings were hatched at the University of Bergen and copepodites were shipped to NIVA. Eleven fish were netted from a 1000 litre fibre-glass tank and respectively infested with approximately 100 copepodites. Infestation lasted for 30 minutes and was carried out in a 40 litre tank with constant aeration of the water. The fish were then subsequently returned to the holding tank. 30 days later all fish were anesthetized and pre-adult lice were removed from the fish until between 16 and 20 parasites remained on each

of the respective fish. The fish were subsequently treated with deltamethrin using the same protocol as described for LS Fr. 16 days later a number of the remaining parasites were removed from the fish; 25 adult females and 7 adult males.

Ten adult female salmon lice from Ls A were collected for the current study. In 2011 Ls A was originally collected in the northern part of Northern Norway and subsequently preserved in a continuous culture at NIVA. These salmon lice had not been exposed to any selection pressure and their deltamethrin sensitivity had been previously documented by Helgesen and Horsberg (2013). An overview of the salmon louse strains and the selection treatments are presented in table 1.

2.5 RNA isolation

Total RNA was extracted from 56 adult female salmon lice (Ls Fr=5, Ls V=15, Ls D=15, Ls D=11 and Ls A=10) using RNeasy Mini kit (Qiagen, CA, USA). RNA was quantified using a ND-100 Spectrophotometer (Thermo Fisher Scientific, DE, USA).

2.6 Quantitative gene expression study

Five lice from each strain (Ls Fr, Ls V, Ls D, Ls Fu and Ls A) were included in the quantitative PCR (qPCR) study. 1 µg RNA from each of these lice were subjected to DNase-treatment using Turbo DNA-free kit (Thermo Fisher Scientific, USA). First strand cDNA was subsequently synthesized from 100-200 ng DNase treated RNA using qScript reverse transcriptase (Quanta Biosciences, USA). This cDNA was then diluted in water to a concentration of 2 ng/µl. 10 ng RNA was used as a PCR template for qPCR employing gene specific primers for each of the three target genes Na_v1.1, Na_v1.2 and Na_v1.3 (outlined below) as well as SsoAdvanced SYBR Green supermix (New England BioLabs, USA), as per manufacturer's protocol. The elongation factor (EF) gene was used as an internal standard/reference gene (Frost and Nilsen, 2003). Two negative controls were employed for each reaction; a non-template control and a non-amplicon control (-RT control). qPCR analysis was carried out in 96 well plates on BioRad CFX96 real-time system. The protocol consisted of denaturation at 95 °C for 30 seconds followed by 40 cycles at 95 °C for 5 seconds and 58 °C for 1 minute. After qPCR, the homogeneity of PCR products was confirmed by melting curve analysis. Each sample was run in triplicates for each of the four genes. The mean Ct values for each gene and sample was calculated and used for normalization of the expression according to the formula $\Delta Ct = Ct_{reference} - Ct_{target}$.

Primers for qPCR:

Na_v1.1 forward: ggctggtccctggcagcgttata

Na_v1.1 reverse: ttcctcagcttgagcggcct

Na_v1.2 forward: cttcccccttgaacggaggtat

Na_v1.2 reverse: cagaaggattctcatgccgaatgt

Na_v1.3 forward: ccgctgaagcaatggaaggaat

Na_v1.3 reverse: gctgttgctatccactcgatcaa

EF forward: ggtcgacagacgtactggtaaacc

EF reverse: tgcggccttggtggtggttc

2.7 Statistical analysis of qPCR data

Analyses of ΔCt -values were performed by repeated analysis of variance (ANOVA), using the statistical software JMP, version 10 (SAS Institute Inc., Cary NC, USA). The factors were gene (Na_v1.1, Na_v1.2 and Na_v1.3), strain (Ls Fr, Ls V, Ls D, Ls Fu and Ls A) and salmon louse number (1-25; as a random effect, nested in strain). The two-way interaction between strain and gene was included in the model. The post hoc test of the gene effect was conducted with the Tukey HSD test. The values presented are the differences in least square (LS) means with 95 percent confidence intervals and

their respective p-values. The post hoc test of the strain effect for each gene was conducted using the Dunnett test with Ls A as the control. The p-values are presented. All results were accepted as significant if $p < 0.05$.

2.8 Sequence analysis

The Na_v gene in *M. domestica* and the $Na_v1.1$ gene in *L. salmonis* were aligned using the previously described alignment. Based on this alignment, sequences of $Na_v1.1$ containing mutation hot spots were selected and specific PCR primers were designed using Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

1 μ g total RNA from each of the respective 56 salmon lice was used for first strand synthesis by qScript reverse transcriptase (Quanta Biosciences, MD, USA).

Reactions were performed using Phusion high-fidelity DNA polymerase (New England BioLabs, USA). PCR was performed under the following conditions: 95 °C for 2.5 min followed by 35 cycles at 95 °C for 30 sec, 59 °C for 30 sec for domain 1 and 40 sec for domain 2 and 3, 72 °C for 1 min and followed by final extension at 72 °C for 10 min. Amplicons were then subjected to direct sequencing, performed by Eurofins MWG Operon, Germany. All sequences were analyzed using Vector NTI software package.

Primers for PCR:

Domain 1:

Forward-primer (1): catgactcaagatctttggaag

Reverse-primer (1): ctgaagatcggatgtgcaac

Domain 2:

Forward-primer (2a): ttgtgttgggactgttgtt

Reverse-primer (2a): cggatcaatgttgcacgta

Forward-primer (2b): cccacactgaactgtctcat

Reverse-primer (2b): gagggaatgatgtcgaatgg

Domain 3:

Forward-primer (3a): tctcgaatagggggaatgaa

Forward-primer (3b): tcgaatagggggaatgaaag

Reverse-primer (3): gcttgaggttccacttgg

3. Results

3.1 Sodium channel genes

The cDNA sequence of $Na_v1.1$ was 97.6 percent identical to one of the three Na_v -genes identified from the salmon louse genome. These were therefore considered to be the same gene. The cDNA sequence from $Na_v1.1$, obtained from RACE PCR, was used for further studies. The nucleotide sequence from the other two identified Na_v -genes was respectively 51 percent ($Na_v1.2$) and 56.1 percent ($Na_v1.3$) identical to $Na_v1.1$.

The cDNA from the $Na_v1.1$ gene in *L. salmonis* has an open reading frame of 5859 base pairs (bp), which encodes a putative protein consisting of 1953 amino acids. The 5' untranslated region (UTR) consists of 320 bp, while the 3' UTR consists of 403 bp. The predicted cDNA sequences of $Na_v1.2$ and $Na_v1.3$ were 4930 bp and 5745 bp, respectively. The adherent putative proteins consist of 1591 and 1915 amino acids, respectively. The putative protein of $Na_v1.3$ does not contain a stop codon, indicating that the predicted cDNA sequence lacks the 3' end of the gene.

The genomic organization revealed that the Na_v1.1 gene spanned approximately 155.2 kb. The first intron, located in the 5'-UTR region of the gene, was very large (87.1 kbp). The gene had 28 exons ranging from 15 to 701 bp. The median exon size was 158 bp. The Na_v1.2 gene spanned 16.3 kbp and consisted of 18 exons with a median exon size of 199 bp. The Na_v1.3 gene spanned 11.3 kbp and consisted of 14 exons, with a median exon size of 305 bp. The genomic organization is presented in figure 1.

The alignment between the deduced amino acid sequences of the *L. salmonis* genes, Na_v1.1, Na_v1.2 and Na_v1.3, and the previously published Na_v genes from insects, arachnida and vertebrates showed a 63.9 to 66 percent identity between Na_v1.1 and the included insect genes. The other two Na_v-genes were 53.1 to 55 percent identical to Na_v1.1 and to the insects' sodium channel genes. Na_v1.2 and Na_v1.3 were 64.8 percent identical to each other. Na_v1.1 was 48 percent and 47 percent identical to the Na_v genes of *D. rerio* and *R. norvegicus*, respectively. Na_v1.2 and Na_v1.3 were between 42.3 and 45.1 percent identical to the aforementioned vertebrate genes. Figure 2 shows an alignment between the *L. salmonis* Na_v-genes and *M. domestica*. When the transmembrane segments' deduced amino acids were aligned separately, identity between Na_v1.1 and *M. domestica* increased to 83.8 percent. Between Na_v1.1 and Na_v1.2 and Na_v1.3 the identity increased to 69.6 and 70.3 percent respectively.

The gene Na_v1.1 cDNA was 99.9 percent identical to the partial cDNA-sequence of a formerly published *L. salmonis* Na_v gene (GB AJ812299.1). Identity between this sequence and the Na_v1.2 and Na_v1.3 genes was 56.4 and 53.8 percent, respectively.

The phylogenetic tree was constructed using the maximum likelihood method on the nucleotide sequences of the three *L. salmonis* Na_v genes and other Na_v genes from 13 different species deposited in GenBank. The phylogenetic tree showed that all salmon louse genes clustered with the acari *T. cinnabarinus*. The other included Na_v gene from a crustacean, *C. borealis*, clustered together with the vertebrates and is a sister group to the insects. The phylogenetic analysis suggests that the three *L. salmonis* Na_v genes have occurred as a result of copepod specific gene-duplications giving rise to three genes (see Fig 3).

3.2 qPCR study

The average normalized expression, $\Delta Ct = Ct_{reference} - Ct_{target}$, with standard deviation was for Na_v1.1: -10.1 (0.7), for Na_v1.2: -14.4 (1.5) and for Na_v1.3: -13.0 (0.5). The ANOVA showed a significant difference between the expressions of the three genes. Main effect of gene: $F_{(2,40)}=244.8$; $p<0.0001$. The post hoc test for the gene effect using Tukey HSD showed significant differences between all genes. All p-values were below 0.001. The difference in least square means (with 95 percent confidence intervals) between the expression of Na_v1.1 and Na_v1.2 was 4.24 (3.77-4.72), between Na_v 1.1 and Na_v1.3 was 2.89 (2.41-3.36) and between Na_v1.2 and Na_v1.3 was 1.36 (0.88-1.83).

The individual normalized expression of the three genes in all parasites is presented in Figure 4. Only a slight difference in average expression between the strains was observed. The average ΔCt value for Na_v1.1 differed by 1.2 between the strains, for Na_v1.2 by 2.6, and by 0.9 for Na_v1.3. There was however some significant differences between the strains. Main effect of strain: $F_{(4,20)}=4.2678$; $p=0.0117$. Main effect of strain x gene: $F_{(8,40)}=4.3551$; $p=0.0008$. Main effect of individual (as a random variable and nested in strain): $F_{(20,40)}=2.0057$; $p=0.0302$. The post hoc test, on the strain effect on each gene, using Dunnett with Ls A as a control, revealed no significant differences between gene expression in Ls A and the other strains for Na_v1.1 and Na_v1.3. The p-values were 0.66 and 0.99 for Ls D, 0.08 and 0.93 for Ls Fr, 0.99 and 0.94 for Ls Fu and 1.00 and 0.99 for Ls V. Though Ls Fr and Ls V expressed significantly more Na_v1.2 than Ls A (p-values <0.001 and 0.0023

respectively), no significant difference was observed between Ls A and Ls D and Ls Fu (p-values of 0.14 and 1.00, respectively).

No expression of the genes was found in the non-template controls or the non-amplicon controls.

3.3 Screening for mutations/polymorphisms

The Na_v1.1 cDNA sequence from *L. salmonis* was aligned with the three sequences from domain I, II and III included in the sequencing study, from 56 different salmon lice. The sequences included in the study are highlighted in figure 2. No base changes leading to amino acid changes were found in the resistant or sensitive samples. However three synonymous single nucleotide polymorphisms (SNP) were identified. Two in domain I: One SNP (ttc and ttt) was at base 1712 (according to the numbering in the *L. salmonis* Na_v1.1 gene). Both codons code for phenylalanine. The other SNP (gat and gac) was at base 1730, both coding for aspartate. The last SNP (atc and att) was in domain III, at base 4622, both coding for isoleucine. The proportions of heterozygote and homozygote samples for each SNP are given in table 2.

4. Discussion

The identity between the deduced amino acid sequence from Na_v1.1 and the other fully sequenced Na_v genes was substantial, with up to 66.1 and 65.9 percent identity to *A. dorsata* and *L. bostrychophila* Na_v genes respectively. There was a greater difference between the other two Na_v genes in *L. salmonis* and both published Na_v genes from other species and the Na_v1.1 gene from *L. salmonis*. Highly conserved amino acid sequences in the four homologous domains of the gene, (I-IV) (Loughney et al., 1989; Wang et al., 2003), were identified in the Na_v genes of the salmon louse. Each domain contained six transmembrane segments, although the segments IVS4 and IVS6 were absent in Na_v1.2 while three segments IS2, IS3 and IVS1 were absent in Na_v1.3. An alignment, of the transmembrane sequences of *L. salmonis* Na_v genes and the Na_v gene of *M. domestica*, revealed that their identity increased when only these segments were aligned.

Similar to observations in other sodium channels; S4, in the three Na_v genes from *L. salmonis* contained repeated motifs of positively charged amino acids, mainly arginine, at every third residue. The two intermediate residues were held by mainly hydrophobic amino acids. This phenomenon was described in *Electrophorus electricus* in 1986 (Guy and Seetharamulu, 1986) where the amino acid order is postulated to give an α -helix surrounded by a spiral ribbon of positive charge. Furthermore the amino acids order is responsible for the voltage gating of the sodium channel by movement of the segment through a narrow channel in each domain (Yang et al., 1996). A hydrophobic triad of isoleucine, phenylalanine and methionine (IFM) in the linker between domain III and IV from rat brain type IIA sodium channel, was found to be critical for rapid inactivation of the channel (West et al., 1992). A homologous motif was present in *L. salmonis*. Similar to other arthropods, such as *M. domestica* and *D. melanogaster*, isoleucine was exchanged with methionine in Na_v1.1, thereby maintaining a hydrophobic triad. In Na_v1.2 and Na_v1.3, isoleucine was replaced with alanine which is also a hydrophobic amino acid.

The four amino acids aspartic acid (D), glutamic acid (E), lysine (K) and alanine (A), one amino acid from each of the P-regions that links segment 5 and 6 in domain I to IV, comprise the DEKA motif. This motif was found in the salmon louse Na_v genes at homologous sites to the DEKA motif in *R. norvegicus* skeletal muscle α subunit (GB Y17153.1). This motif has proven crucial for the ionic selectivity of the sodium channel (Heinemann et al., 1992; Sun et al., 1997). Channels with DEKA motif as their ion selectivity filter are termed Na_v1 (Barzilai et al., 2012) and hence the *L. salmonis* three genes were called Na_v1.1, Na_v1.2 and Na_v1.3.

Phosphorylation of the sodium channel gene by cAMP-dependent protein kinase A (PKA) at sites in the intracellular linker between domains I and II and by protein kinase C (PKC) in the intracellular

linker between domain III and IV, to regulate the activity of the channel, is a known phenomenon in vertebrate sodium channels (Catterall, 1992). Several potential PKA phosphorylation sequences were present in the linker between domain I and II in the *L. salmonis* Na_v genes. One potential PKC phosphorylation sequence was found in the linker between domain III and IV in Na_v1.1 and two were identified in Na_v1.2 and Na_v1.3. These corresponded to the amino acid consensus sequences presented by Pearson and Kemp (1991). Further studies are necessary in order to establish whether these regulating mechanisms are present in salmon lice sodium channels or not.

These features, described above from the Na_v1 genes are all in accordance with other published Na_v1 genes, with the exception of the five lacking segments, from Na_v1.2 and Na_v1.3, which were unexpected. These two genes were, however, only predicted from the salmon louse genome and the postulation of exons and introns may be slightly erroneous. In order to fully elucidate the gene sequence of Na_v1.2 and Na_v1.3, RACE PCR should be performed. The findings of three different Na_v genes in *L. salmonis* where in accordance with the transcriptome analysis, conducted in the copepod *Calanus finmarchicus*, where three putative Nav1 genes were identified (Lenz et al., 2014). Functional studies are however required to substantiate that the three Na_v1 genes in *L. salmonis* encode functional Na_v channels. The study by Lenz et al. (2014) also detected a Na_v2 gene in *C. finmarchicus*, characterized by a DEEA selectivity filter pattern. One gene with the same selectivity filter pattern was also found in the *L. salmonis* genome, indicating the presence of a Na_v2 gene in this copepod as well.

The modelled pyrethroid binding site in the sodium channel gene has hydrophobic residues that differ between arthropods and non-arthropods (O'Reilly et al., 2006). These differences are considered to contribute to the specificity of these chemicals. Mammalian sodium channels are 1000-times less sensitive to pyrethroids than the sodium channels in arthropods (Vais et al., 2001). The *L. salmonis* Na_v1 genes have the same amino acids at these positions as other arthropods with the exception of the IIS6 position where Na_v1.2 has alanine as opposed to phenylalanine. According to the model by O'Reilly (2006), pyrethroids stabilize the IIS4-S5 linker and the IIS5 and IIS6 in an activated conformation. This may clarify why mutations in these sequences can result in pyrethroid resistance.

Mutations in Na_v1 genes in other arthropods, with known association to pyrethroid resistance, were selected through literature studies (O'Reilly et al., 2006; Rinkevich et al., 2013). These mutations are numbered, based on the *M. domestica* Na_v1 gene (Williamson et al., 1996). Homologous sequences to known mutation hot spots, in pyrethroid resistant arthropods, were chosen for screening. A comparison, of these sequences in salmon lice with sequences from sensitive arthropods, showed that the salmon louse genes contained the same amino acids at the homologous positions. The only exception was at position 410 in Na_v1.2 and Na_v1.3 which had the amino acid isoleucine instead of valine. Both of these amino acids are however highly hydrophobic, non-polar and neutrally charged.

The Na_v1.1 gene was chosen for mutation screening based on the predominant expression of this gene in comparison to the other two Na_v1 genes. This overexpression was shown by the current qPCR study. Furthermore these results were also supported by transcriptome data collected from LiceBase, where the counts per million reads from adult female lice from Na_v1.1, Na_v1.2 and Na_v1.3 were 5.2, 0.6 and 0.4, respectively. The qPCR study gave no indication with regard to changes in expression of the three genes as a possible cause of resistance. However two of the salmon louse strains, Ls Fr and Ls V, had a significantly higher expression of Na_v1.2 compared to the sensitive strain Ls A. The expression of this gene in the two other resistant strains (Ls D and Ls Fu) was, however, not significantly different from the sensitive strain (Ls A). Given that resistance is most probably caused by the same mechanism in all strains, the overexpression of Na_v1.2 in Ls Fr and Ls V could be an interstrain variation and do not appear to be associated to resistance. The theory with a common resistance mechanism causing a specific type of resistance has been shown as probable for

emamectin benzoate resistance in *L. salmonis*, throughout the Atlantic Ocean, in a study by Besnier et al. (2014).

A 99.9 percent identity between the partial cDNA-sequence from a *L. salmonis* Na_v gene, previously described by Fallang et al. (2005), and the gene Na_v1.1 presented in the current study strongly indicates it to be the same gene. The two bases that comprise the 0.1 percent difference between Na_v1.1 and the previously published sequence were identical in the full gene cDNA sequence and in all 56 salmon lice screened for mutations, including both resistant and sensitive lice. These differences were therefore not considered to have any association with resistance.

No mutations were found in the current study in any of the regions screened. This includes the three segments modelled by O'Reilly et al. (2006) to be altered by pyrethroid exposure. Unexpectedly the mutation found by Fallang et al. (2005) was not found in any of the samples, indicating that it does not play a significant role in pyrethroid resistance. Furthermore, this study strongly suggests that pyrethroid resistance in salmon lice is not primarily caused by mutations in the Na_v1.1 gene. This is due to the fact that all residues, with known association to pyrethroid resistance from more than one species, as reviewed by Rinkevich et al. (2013), were included in the current resistance study. Residues with functionally proven association to resistance, as reviewed by O'Reilly et al. (2006) were also screened. Moreover, the two known residues with pyrethroid resistance associated mutations in a crustacean, *Hyalella azteca*, were also covered in the current study (Weston et al., 2013).

However, some of the mutations associated with pyrethroid resistance in a Na_v1 gene as shown in Davies et al. (2007), were not included in the current study. The heterogeneity in the single nucleotide polymorphisms amongst the resistant parasites observed in the current study, both within and between salmon louse strains, contradicts the concurrent presence of mutations. This is due to the fact that a possible mutation in the gene would be expected to have been originated from a single site and subsequently spread along the coast. This was the case for emamectin benzoate resistant salmon lice (Besnier et al., 2014). Despite the fact that mutations in the sodium channel gene in other species have been shown to have multiple origins in the same species, the independent haplotypes have revealed a geographical distribution (Pinto et al., 2007; Franck et al., 2012; Rinkevich et al., 2012).

Another possible explanation, for the absence of mutations in Na_v1.1 in salmon lice, is the absence of resistance in the study material. This was most likely the case in the study of human scabies mites by Andriantsoanirina et al. (2014). However, given that the samples in the current study were chosen from four different parts of Norway with known pyrethroid resistance, both from field treatments and field bioassay results, this hypothesis is highly unlikely. Furthermore, with the exception of the sensitive strain, all transcript-analysis subjected parasites survived a deltamethrin treatment carried out according to the producer's recommendations.

Given that two of the three Na_v1 genes were excluded from the present mutation study, the possibility exists that mutations in these genes might have resulted in pyrethroid resistance in salmon lice. However, this possibility seems unlikely given that these genes were expressed at a significantly lower level than the Na_v1.1 gene. Furthermore the various Na_v genes would be expected to have been exposed to the same selection pressure and therefore should have shown similar resistance mechanisms. This was seen in Na_v genes in the tetrodotoxin resistant snake, *Thamnophis sirtalis*. In this snake, all the three paralogs of Na_v1 genes, from skeletal muscle and the peripheral nervous system, contained resistance-causing mutations while Na_v1 paralogs protected by the blood-brain barrier did not have these same mutations (McGlothlin et al., 2014). The distribution of the different Na_v1 channels in *L. salmonis* is however not known. Hence, to comment on this aspect is beyond the current study.

Resistance mechanisms outside of the sodium channels may provide a fourth possible explanation for the absence of target-site mutations in pyrethroid resistant salmon lice. Metabolic resistance without any target-site mutations was found in pyrethroid resistant *A. gambia* as well as in various Anopheles species (Wondji et al., 2007; Verhaeghen et al., 2009). In these two studies, cytochrome P450s and esterases were suggested to be the cause of resistance. Furthermore overexpression of glutathione S-transferases and superoxide dismutases also have been connected to pyrethroid resistance in arthropods (Vontas et al., 2001; Müller et al., 2007). Finally P450 monooxygenases have been suggested to be involved in pyrethroid resistance in salmon lice (Sevatdal et al., 2005b). All of these observations warrant further studies on the metabolic resistance as a plausible mechanism in pyrethroid resistance in salmon lice.

Results, from the present study suggest that mutations in the voltage gated sodium channel genes are not associated with pyrethroid resistance in Norway. However, the possibility of Na_v1 genes playing a role in pyrethroid resistance in salmon lice in other countries cannot be excluded. Target-site mutations and metabolic resistance have been observed in other pyrethroid resistant arthropods as both dual cause and single cause in the same species (Devonshire et al., 1998).

To conclude our findings, there is a large degree of homology between the genes described in the current study and the previously described Na_v1 genes from other arthropods. All three genes contain several features characteristics of sodium channel genes and are thus presumed to be the Na_v1 genes in *L. salmonis*. The present genes may therefore be applied in future functional studies. The Na_v1.1 gene was the predominantly expressed gene among the three Na_v1-genes in *L. salmonis*. Changes in the expression of the Na_v1-genes were not seen to be associated with resistance. Mutations in the Na_v1.1 gene were not identified in pyrethroid resistant salmon lice from four different locations in Norway. Hence, target-site mutations in this gene are presumably not involved in pyrethroid resistance in Norwegian salmon lice. However, further studies are required to confirm our findings. Nevertheless, based on our observations, studies on pyrethroid resistance mechanisms should be targeted at different forms of metabolic resistance.

Acknowledgement

The current study was a joint effort between the projects: Prevent (NFR 199778/S40) and the Sea Lice Research Centre (NFR 203513/030) platform, both funded by the Norwegian Research Council with additional funding from The Norwegian Seafood Research Fund for Prevent. We would also like to thank the fish health services who contributed in collecting the parasites: Helgeland Havbruksstasjon AS, Aqua kompetanse AS and Havbrukstjenesten AS. In addition thanks to Josephine Prendergast for proofreading the manuscript.

Reference list

- Andriantsoanirina, V., Izri, A., Botterel, F., Foulet, F., Chosidow, O., Durand, R., 2014. Molecular survey of knockdown resistance to pyrethroids in human scabies mites. *Clin. Microbiol. Infec.* 20, O139-O141.
- Barzilai, M.G., Reitzel, A.M., Kraus, J.E.M., Gordon, D., Technau, U., Gurevitz, M., Moran, Y., 2012. Convergent evolution of sodium ion selectivity in metazoan neuronal signaling. *Cell Rep.* 2, 242-248.
- Besnier, F., Kent, M., Skern-Mauritzen, R., Lien, S., Malde, K., Edvardsen, R.B., Taylor, S., Ljungfeldt, L.E., Nilsen, F., Glover, K.A., 2014. Human-induced evolution caught in action: SNP-array reveals rapid amphi-atlantic spread of pesticide resistance in the salmon ectoparasite *Lepeophtheirus salmonis*. *BMC Genomics.* 15.
- Bloomquist, J.R., 1996. Ion channels as targets for insecticides. *Annu. Rev. Entomol.* 41, 163-190.
- Bravo, S., Nuñez, M., Silva, M.T., 2013. Efficacy of the treatments used for the control of *Caligus rogercresseyi* infecting Atlantic salmon, *Salmo salar* L., in a new fish-farming location in Region XI, Chile. *J. Fish. Dis.* 36, 221-228.
- Catterall, W.A. 1992. Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev.* 72, S15-S48.
- Davies, T.G.E., Field, L.M., Usherwood, P.N.R., Williamson, M.S., 2007. DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB Life.* 59, 151-162.
- Devonshire, A.L., Field, L.M., Foster, S.P., Moores, G.D., Williamson, M.S., Blackman, R.L., 1998. The evolution of insecticide resistance in the peach-potato aphid, *Myzus persicae*. *Philos. T. Roy. Soc. B.* 353, 1677-1684.
- Fallang, A., Denholm, I., Horsberg, T.E., Williamson, M.S., 2005. Novel point mutation in the sodium channel gene of pyrethroid-resistant sea lice *Lepeophtheirus salmonis* (Crustacea: Copepoda). *Dis. Aquat. Organ.* 65, 129-136.
- Franck, P., Siegwart, M., Olivares, J., Toubon, J.F., Lavigne, C., 2012. Multiple origins of the sodium channel kdr mutations in codling moth populations. *Plos One.* 7.
- Frost, P., Nilsen, F., 2003. Validation of reference genes for transcription profiling in the salmon louse, *Lepeophtheirus salmonis*, by quantitative real-time PCR. *Vet. Parasitol.* 118, 169-174.
- Grave, K., Horsberg, T.E., Lunestad, B.T., Litleskare, I., 2004. Consumption of drugs for sea lice infestations in Norwegian fish farms: methods for assessment of treatment patterns and treatment rate. *Dis. Aquat. Organ.* 60, 123-131.
- Guy, H.R., Seetharamulu, P., 1986. Molecular model of the action potential sodium channel. *Proc. Natl. Acad. Sci.* 83, 508-512.
- Hamre, L.A., Glover, K.A., Nilsen, F. 2009. Establishment and characterisation of salmon louse (*Lepeophtheirus salmonis* (Krøyer 1837)) laboratory strains. *Parasitol. Int.* 58, 451-460
- Hart, J.L., Thacker, J.R., Braidwood, J.C., Fraser, N.R., Matthews, J.E., 1997. Novel cypermethrin formulation for the control of sea lice on salmon (*Salmo salar*). *Vet. Rec.* 140, 179-181.

- Heinemann, S.H., Terlau, H., Stuhmer, W., Imoto, K., Numa, S., 1992. A single mutation alters the sodium-channel to a calcium-permeable channel. *FASEB J.* 6, A133.
- Helgesen, K.O., Bravo, S., Sevatdal, S., Mendoza, J., Horsberg, T.E., 2014. Deltamethrin resistance in the sea louse *Caligus rogercresseyi* (Boxhall and Bravo) in Chile: bioassay results and usage data for antiparasitic agents with references to Norwegian conditions. *J. Fish. Dis.* 37, 877-890.
- Helgesen, K.O., Horsberg, T.E., 2013. Single-dose field bioassay for sensitivity testing in sea lice, *Lepeophtheirus salmonis*: development of a rapid diagnostic tool. *J. Fish. Dis.* 36, 261-272.
- Jones, S., Beamish, R., 2011. Salmon lice. An integrated approach to understanding parasite abundance and distribution. John Wiley & Sons, Inc., Chichester.
- Lenz, P.H., Roncalli, V., Hassett, R., Wu, L.S., Cieslak, M.C., Hartline, D.K., Christie, A.E., 2014. *De novo* assembly of a transcriptome for *Calanus finmarchicus* (Crustacea, Copepoda) - the dominant zooplankton of the North Atlantic Ocean. *Plos One.* 9.
- Loughney, K., Kreber, R., Ganetzky, B., 1989. Molecular analysis of the para locus, a sodium-channel gene in drosophila. *Cell.* 58, 1143-1154.
- McGlothlin, J.W., Chuckalovcak, J.P., Janes, D.E., Edwards, S.V., Feldman, C.R., Brodie, E.D., Pfrender, M.E., Brodie, E.D., 2014. Parallel evolution of tetrodotoxin resistance in three voltage-gated sodium channel genes in the garter snake *Thamnophis sirtalis*. *Mol. Biol. Evol.* 31, 2836-2846.
- Müller, P., Donnelly, M.J., Ranson, H., 2007. Transcription profiling of a recently colonised pyrethroid resistant *Anopheles gambiae* strain from Ghana. *BMC Genomics.* 8.
- O'Reilly, A.O., Khambay, B.P.S., Williamson, M.S., Field, L.M., Wallace, B.A., Davies, T.G.E., 2006. Modelling insecticide-binding sites in the voltage-gated sodium channel. *Biochem. J.* 396, 255-263.
- Pearson, R.B., Kemp, B.E., 1991. Protein-kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol.* 200, 62-81.
- Pinto, J., Lynd, A., Vicente, J.L., Santolamazza, F., Randle, N.P., Gentile, G., Moreno, M., Simard, F., Charlwood, J.D., do Rosario, V.E., Caccone, A., della Torre, A., Donnelly, M.J., 2007. Multiple origins of knockdown resistance mutations in the afro-tropical mosquito vector *Anopheles gambiae*. *Plos One.* 2.
- Ranson, H., N'Guessan, R., Lines, J., Moiroux, N., Nkuni, Z., Corbel, V., 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol.* 27, 91-98.
- Rinkevich, F.D., Du, Y.Z., Dong, K., 2013. Diversity and convergence of sodium channel mutations involved in resistance to pyrethroids. *Pestic. Biochem. Physiol.* 106, 93-100.
- Rinkevich, F.D., Hedtke, S.M., Leichter, C.A., Harris, S.A., Su, C., Brady, S.G., Taskin, V., Qiu, X.H., Scott, J.G., 2012. Multiple origins of kdr-type resistance in the house fly, *Musca domestica*. *Plos One.* 7.
- Roth, M. 2000. The availability and use of chemotherapeutic sea lice control products. *Contrib. Zool.* 69, 109-118.

- Sevatdal, S., Copley, L., Wallace, C., Jackson, D., Horsberg, T.E., 2005a. Monitoring of the sensitivity of sea lice (*Lepeophtheirus salmonis*) to pyrethroids in Norway, Ireland and Scotland using bioassays and probit modelling. *Aquaculture*. 244, 19-27.
- Sevatdal, S., Fallang, A., Ingebrigtsen, K., Horsberg, T.E., 2005b. Monooxygenase mediated pyrethroid detoxification in sea lice (*Lepeophtheirus salmonis*). *Pest. Manag. Sci.* 61, 772-778.
- Sevatdal, S., Horsberg, T.E., 2000. The identification of pyrethroid resistance in salmon lice (in Norwegian: Kartlegging av pyretroidresistens hos lakselus). *Norsk fiskeoppdrett*. 12, 34-35.
- Sevatdal, S., Horsberg, T.E., 2003. Determination of reduced sensitivity in sea lice (*Lepeophtheirus salmonis* Krøyer) against the pyrethroid deltamethrin using bioassays and probit modelling. *Aquaculture*. 218, 21-31.
- Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W.Z., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7.
- Sun, Y.M., Favre, I., Schild, L., Moczydlowski, E., 1997. On the structural basis for size-selective permeation of organic cations through the voltage-gated sodium channel. Effect of alanine mutations at the DEKA locus on selectivity, inhibition by Ca²⁺ and H⁺, and molecular sieving. *J. Gen. Physiol.* 110, 693-715.
- Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial-DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10, 512-526.
- Tamura, K., Stecher, G., Peterson, D., Filipowski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 30, 2725-2729.
- Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O.T., Nilsen, F., Horsberg, T.E., Jackson, D., 2013. Salmon lice - impact on wild salmonids and salmon aquaculture. *J. Fish. Dis.* 36, 171-194.
- Vais, H., Williamson, M.S., Devonshire, A.L., Usherwood, P.N.R., 2001. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. *Pest Manag. Sci.* 57, 877-888.
- Vais, H., Williamson, M.S., Goodson, S.J., Devonshire, A.L., Warmke, J.W., Usherwood, P.N.R., Cohen, C.J., 2000. Activation of *Drosophila* sodium channels promotes modification by deltamethrin. Reductions in affinity caused by knock-down resistance mutations. *J. Gen. Physiol.* 115, 305-318.
- Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W., Tirry, L., 2010. Acaricide resistance mechanisms in the two-spotted spider mite *Tetranychus urticae* and other important Acari: A review. *Insect Biochem. Mol. Biol.* 40, 563-572.
- Verhaeghen, K., Van Bortel, W., Ho, D.T., Sochantha, T., Coosemans, M., 2009. Absence of knockdown resistance suggests metabolic resistance in the main malaria vectors of the Mekong region. *Malar. J.* 8.
- Vontas, J.G., Small, G.J., Hemingway, J., 2001. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochem. J.* 357, 65-72.
- Wang, R.W., Huang, Z.Y., Dong, K., 2003. Molecular characterization of an arachnid sodium channel gene from the varroa mite (*Varroa destructor*). *Insect Biochem. Mol. Biol.* 33, 733-739.

- Wang, R.W., Liu, Z.Q., Dong, K., Elzen, P.J., Pettis, J., Huang, Z.Y., 2002. Association of novel mutations in a sodium channel gene with fluvalinate resistance in the mite, *Varroa destructor*. J. Apicult. Res. 41, 17-25.
- West, J.W., Patton, D.E., Scheuer, T., Wang, Y.L., Goldin, A.L., Catterall, W.A., 1992. A cluster of hydrophobic amino-acid-residues required for fast Na(+)-channel inactivation. Proc. Natl Acad. Sci. USA. 89, 10910-10914.
- Weston, D.P., Poynton, H.C., Wellborn, G.A., Lydy, M.J., Blalock, B.J., Sepulveda, M.S., Colbourne, J.K., 2013. Multiple origins of pyrethroid insecticide resistance across the species complex of a nontarget aquatic crustacean, *Hyalella azteca*. Proc. Natl Acad. Sci. USA. 110, 16532-16537.
- Whyte, S.K., Westcott, J.D., Jimenez, D., Revie, C.W., Hammell, K.L., 2014. Assessment of sea lice (*Lepeophtheirus salmonis*) management in New Brunswick, Canada using deltamethrin (AlphaMax®) through clinical field treatment and laboratory bioassay responses. Aquaculture 422-423, 54-62.
- Williamson, M.S., Martinez-Torres, D., Hick, C.A., Castells, N., Devonshire, A.L., 1996. Analysis of sodium channel gene sequences in pyrethroid-resistant houseflies. Progress toward a molecular diagnostic for knockdown resistance (kdr). In: Brown, T.M., Molecular genetics and evolution of pesticide resistance. American Chemical Society, Washington D.C., pp. 52-61.
- Wondji, C.S., Morgan, J., Coetzee, M., Hunt, R.H., Steen, K., Black, W.C., Hemingway, J., Ranson, H., 2007. Mapping a quantitative trait locus (QTL) conferring pyrethroid resistance in the African malaria vector *Anopheles funestus*. BMC Genomics. 8.
- Xu, L., Wu, M., Han, Z.J., 2013. Overexpression of multiple detoxification genes in deltamethrin resistant *Laodelphax striatellus* (Hemiptera: Delphacidae) in China. Plos One. 8.
- Yang, N.B., George, A.L., Horn, R., 1996. Molecular basis of charge movement in voltage-gated sodium channels. Neuron. 16, 113-122.

Table 1

The salmon lice material used in screening for target-site mutations in the Na_v1.1 gene in pyrethroid resistant *L. salmonis* is presented. Resistance status was based on results from laboratory bioassays and small-scale treatment for LS A and on field treatment results for the other four salmon lice strains. The time of infestation, deltamethrin treatment and salmon lice collection is given. Details of the deltamethrin treatment are given in the text.

Salmon louse strain	Resistant status	Origin	Time of infestation	Treatment	Collection date
Ls A	Sensitive	Northern part of Northern Norway	2011	No	2013
Ls Fr	Resistant	South of Mid-Norway	45 lice 20/3-13	26/3-13	7 lice 3/4-13
Ls D	Resistant	South of Northern Norway	40 lice 3/5-13	7/5-13	21 lice 14/5-13
Ls V	Resistant	North of Mid-Norway	80 lice 19/4-13	23/4-13	34 lice 30/4-13
Ls Fu	Resistant	South-west coast of Norway	copepodites 13/9-13	14/10-13	32 lice 5/11-13

Table 2

The number of mutations found in Na_v1.1 for each domain and for each salmon lice strain is shown. The figure also presents the distribution of three single nucleotide polymorphisms (SNPs) in each strain. The SNPs were numbered (1712, 1730 and 4622) based on the nucleotide number in the Na_v1.1 gene in salmon lice. cc: homozygote for cytosine, ct: heterozygote, tt: homozygote for thymine.

Salmon lice		Mutations			Single nucleotide polymorphisms		
					cc/ct/tt		
Strain	n	Domain 1	Domain 2	Domain 3	1712	1730	4622
Ls A	10	0	0	0	0/0/10	10/0/0	0/0/10
Ls D	15	0	0	0	6/5/4	4/7/4	6/4/5
Ls V	15	0	0	0	5/6/4	4/6/5	5/6/4
Ls Fr	5	0	0	0	2/2/1	2/2/1	2/3/0
Ls Fu	11	0	0	0	2/7/2	3/6/2	1/7/3
Total	56						

Legends to figures

Figure 1

Genomic organisation of the voltage gated sodium channel genes in the *L. salmonis* genome. The Na_v1.1 gene spanned 155.2 kilobase pair (kbp). The first intron, located in the 5'-UTR region of the gene, was extremely large (87.1 kbp). It had 28 exons ranging from 15 to 701 bp. The cDNA sequence consisted of 5508 bp with a median exon size of 158 bp. The Na_v1.2 gene spanned 16.3 kbp and consisted of 18 exons with a median exon of 199 bp. The predicted cDNA sequence was 4930 bp. The Na_v1.3 gene spanned 11.3 kbp and consisted of 14 exons with a median exon of 305 bp. The predicted cDNA sequence was 5745 bp.

Figure 2

Alignment of the deduced amino acid sequences of the voltage gated sodium channels in *Lepeophtheirus salmonis*; Na_v1.1 (1.1), Na_v1.2 (1.2) and Na_v1.3 (1.3), as well as *Musca domestica* (M.d) (GenBank X96668.1). All six segments from all four domains in the gene are underlined. The sequences screened for mutations in Na_v1.1 in the current study are presented in bold letters. All covered hot spots for mutations from other arthropods are shown in boxes. The numbering on top of the boxes represents the amino acid number in *M. domestica*. The DEKA-motif is shown by four letter labeled boxes.

Figure 3

The phylogenetic tree was constructed using the nucleotide sequences from the sodium channel genes in *L. salmonis* and 13 other vertebrate and invertebrate species (gene sequences collected from GenBank). The tree was constructed using the maximum likelihood method. Credibility of internal branch was evaluated using bootstrapping method (numbers are given in percent on each branch). The tree is drawn to scale, with branch lengths measured by the number of substitutions per site.

Figure 4

The results from the quantitative PCR study of the three voltage gated sodium permeable channels genes, Na_v1.1, Na_v1.2 and Na_v1.3, in *L. salmonis* are shown in an one dimensional scatterplot. The results from each gene are presented consecutively on the x-axis. 5 adult salmon lice from four pyrethroid resistant strains; Ls Fr, Ls V, Ls D and Ls Fu and one sensitive strain; Ls A, were included in the study. The normalized expression, $\Delta Ct = Ct_{reference} - Ct_{target}$, is displayed on the y-axis for each salmon lice sample. Elongation factor was used as the reference gene. The results from the different louse strains are presented with open circles for Ls Fr, crosses for Ls V, triangles for Ls D, pluss-signs for Ls Fu and filled circles for Ls A.

Figure 1

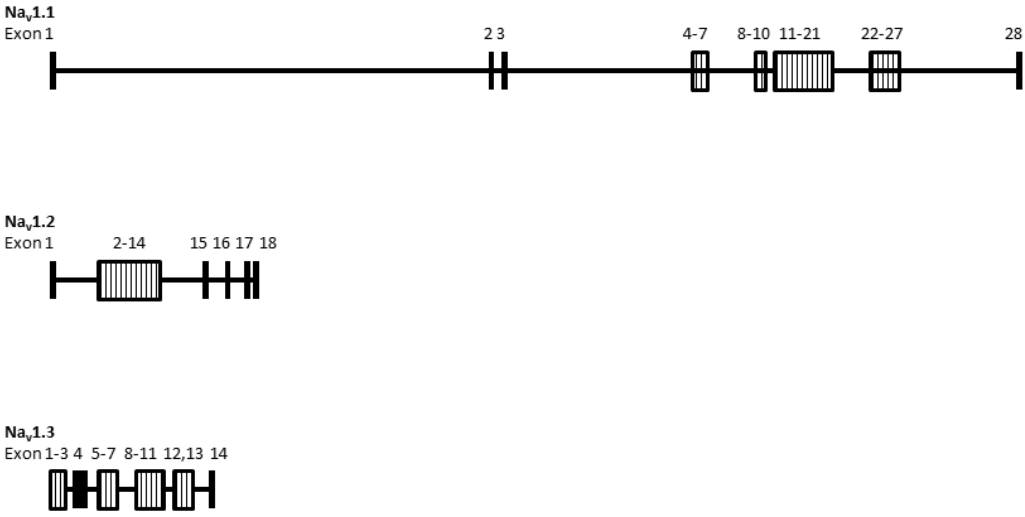


Figure 2

```

1.1 -----MSESDSDSIEDDRPIFVFPFTRESIAKIEARILEDNEKKRELQOKRAE--
1.2 MVHPQAYNSFLSICTMYEIEITGSIPTFRPFTREDEYKIEENRIFEKKLIQKKRDEKRRKNI
1.3 -----MAEADSENSLPTFRPFTREELAIHHRIMEKKLAAKKAERRAKNI
M.d -----MTEDSDSISEERSLFRPFTRESLLQIEQRIAEHEKQKELERKRAE--

1.1 -----GEVRYDDDDDDGPIPDPTLEAGMPLPIRMAAEFPPELVATPIVDI
1.2 KEFGDGARARKQYNQSDDEDDDPESVPIPNKRIEQGNDLPRR-YGEFPLELSNTPICDI
1.3 AEFGDSARARKLYEQDSESESEDEI---EQNPKLEQGNDLPRR-YGEFPLEFASTPICDI
M.d -----GEQIRYDDEDEDEGPQDPDPTLEQGVPIPVRMQGSFPPELASTPLEDI

IS1
1.1 DSFYSNKKTFFIVISKGRDIFRFNAEDALYVLSFPSPVRRVAIHILVHPFFSVFIITITILL
1.2 DPFYADQRTFMVIAKNGSITRFSAPALFLLTPFHPPIRRIAIRVLTHPLFNFIICTILV
1.3 DPFYFRDKKSFIVISKGGTIFRFSANAMFLLSPYHPIRRIAIIHILHTLTLNLMATILV
M.d DPFYSNVLTFVVISKGDIFRFSASKAMWLLDPFNPIRRVAIYILVHPLFSLFIITITILT

IS2 IS3
1.1 NCLMMIKTSNERIESSEVIFTAIYTFESATKVMGRGFILCPFSYLRDAWNWLDIVITLA
1.2 NCIVMMLPDTEYXSASEIIFTSIYTESAVKLLARGFILHNFTYLRDPWNWLDFAVIGMS
1.3 NCYVMIKPDTE-----
M.d NCILMIMPTTPTVESTEVIFTGIYTFESAVKVMARGFILCPFTYLRDAWNWLDVVIALA

IS4 IS5
1.1 YITMGIDLGNLAVLRTFRVLRALKTVAIIPGLKTIVGAVIESVKNLRDVIILTVFSLSVF
1.2 YITIAIDLGSFSLRTRFRVFRALKSVAXVPGLKTIVGAIISVKNLFDVTILTLFGLALF
1.3 -----TFRVFRALKSVAVIPGLKTIVSAIISVKNLRDVIILTMFALSVE
M.d YVTMGIDLGNLALRTRFRVLRALKTVAIIPGLKTIVGAVIESVKNLRDVIILTMFALSVE

1.1 ALLGLQIYMGVLTQKCIHDFPWSIPPEERGNYS SHIAWNKFNLNSSNWWIEGRIDPVL
1.2 ALLGLQLYMGVLTQRCIYEIPDELL---EMNNGSVHSVYKSWYINSSNLYVSEVTNDYIM
1.3 ALLGLQIYMGVLSQKCVYTPSPGSEEFYWGNTAESFHAWYSNKSSWYLS--NDNYIM
M.d ALMGLQIYMGVLTQKCIKRFPLDGSW---G-NLTDENWFLHNSNSNWFTEGSEYPV

D
1.1 CGNSSGAGECPDNYTCLQGFRNPNDYTSFDTFGWAFLSAFRLMTQDLWEDLYQSVLRT
1.2 CGNSSGAGKCEPSTVCLAGFAQNPNGYTTFDNFGYAYLCAFRMLTQDYWENLYQITLRT
1.3 CGNASGAGKCPDGTTCMEGFGPNPNYGYTSFDNFGSAYLCAFRMLTQDFWENLYQITLRT
M.d CGNVSGAGQCCEYVCLQGFQPNPNYDYTSFDSFGWAFLSAFRLMTQDFWEDLYQHVLQA

IS6 410 435
1.1 AGPWHVIFFLVIFLGSFYLVNLLILAIIVAMSYDELQKKAEEEEAAQAEELREAEAAA
1.2 AGPWNILFFILNIFMGSFYLVNLLILAIIVAMSYDELQRE-----LEELEAKAAEIAV
1.3 AGPWHIIFFMCNIFLGSFYLVNLLILAIIVAMSYDELQRLAEEEAQRELELEAIKEAEAAA
M.d AGPWHMLFFIVIIFLGSFYLVNLLILAIIVAMSYDELQKKAEEEEAA---EEEAIREAEAAA

1.1 AIAEMKLCNCFDEEDE---D---ESEEDEKPYEN--DEDELSCTS-----
1.2 FKEAEAL-----GDVSKVNFSS-----
1.3 LAEAEAAAAXAAEAMEGLFGDIDDPGGGGGAVDFPESPARESLVSTSIDR-----VDT
M.d AAKAAKLEERANVAAQ---AAQDAADAAAAALHPEMA--KSPYSCISYELFVGGGKGN

1.1 -----DL-----QHFKKDS-----YSKIIVTPSSVLPED
1.2 -----SSGVDNPSFKFD-----EDVALPFTQS
1.3 QQPPEERMSLRASLSVYR-----SEDSLDEPHNLHRIRQTLNNSKXSLPASPFVRR
M.d DDNNKEKMSIRSVEVESESVSVIQRQPAPTAPATKVRKVS-----TTSLSLPGSPFNLR

1.1 KGMMGRRCPNQHEWHIT-----PNA
1.2 S-----IS---NTS-----NLEFLP
1.3 T-----SKNSFSSHLRLQEKYG-NSASKKPLVLFVDAQEHLPHYADDSAVTPKS
M.d -----RGRSSSHKYTIR-NGRGRFGIPGSDRKLPLVLQTYQDAQQHLPYADDSNAVTPMS

1.1 DTND-----
1.2 PLNGGIEPKLTNHRDLLILKDV-IN--EVSMDQHIRHENPSDYNGR-----
1.3 ELNGGIVVDAPNRINNINSRKYSYNSHTGKIDSYNSHTDLSRYNNNNNGSNHTKEGTLRI
M.d EENGAIIVPAY--YCNLGSRHSSYTSHQSRIS-YTSHGDLGMAAMGASTMTKESKLRS

1.1 -----KVDK-FDDLSSYPKGQPPDFNGTNGV-----HYR-RRSQESYYSNPFISPNE
1.2 -----LNNN-----GFDLRE-----
1.3 RMAS-----LFGDKNNGGPPSSNNSKVLHDPITNQYDLNR-----SSHFINNNHE
M.d RNTRNQSIGAAATNGSSSTAGGGYPDANHKEQRD-YEMGQDYTDEAGKIKHHDNPFIEPVQ

1.1 RHASVNIKDVVLNDIINQASARVTSTIKNPESTRSSRQLERKQREARKISLYLEAKHDE
1.2 -----NESEDED
1.3 RKDSIDIQDVRVLSNIIDQVSDRSMSKATHM-----S-----RYIDDGDADDD
M.d TQTVVDMKDVMLNDIIEQAAGRHSRASE-----RGEDDDE

```

IIS1

1.1 EGRVKEKLVAVDFDKCIDIFCVWDCCWAYVKLTEYLSFIIFDPFVDFITLCLIVNTFFM
1.2 SMTIFKRKGIKVLKQLGEIFCIWDCCVWIRFSEVLAVIVDFPFVDSLITLCIVVNVLFM
1.3 EGLKFKDIFCEYFKKVVNIMCIWDCCWLWIKTSQILAFIVDFPFTELFTICIAVNVIFM
M.d DGPTFKDIALEYILKGEIFCVWDCCVWVWVKFQEWVSFIVDFPFVELFITLCIVVNTMFM

IIS2

1.1 GLDHHD-----MDPDIHKTLQNGNYFFTATFAIESCIKLMAMSPKYFFLEGWNI FDF
1.2 MFDHYNIEYDANGGMSMEMNLLKNGNYFFTIFAFESFMKLMAMSPRYFFADGWNCDFD
1.3 ALDRYDIEYDANGGMSPLSSVLTQGNFYFFTIFAVESFIKLVAMSPRYFFSEGWNCDFD
M.d AMDHHD-----MNPELEKVLKSGNYFFTATFAIEASMKLMAMSPKYFFQEGWNI FDF

IIS3

IIS4

918 925 929 932

1.1 IIVSLSLIELGLVNVSGLTVLRFRLLRVFKLAKSWPTLNLLISIMGKTVGALGNITLVL
1.2 LIVVLSVELLAXGVNGLSMLRSFRLLRVFKLAKSWKSLNDIMTAMANTLGALESLNITLVL
1.3 LIVVLSVELLAEVSGLSMLRSFRLLRVFKLAKSWKSLNDILTAMANTFGALESLNITLVL
M.d IIVALSLLELGLGEGVQGLSVLRSFRLLRVFKLAKSWPTLNLLISIMGRTMGALGNITLVL

IIS5

945

E

1.1 CIIIFIFAVMGMLFGKSYVDNIDRFPNKSLPRWNFVDFMHSFMIVFRVLCGEWIESMWD
1.2 CIIIFIFAVMGMLFGSFYIEKACEKWKCELPRWNFTDFLHFSFMIVFRVLCGEWIESMWA
1.3 CIIIFIFAVMGMLFGKDYDGVCEKWDCEKWDMPRNFTDFLHFSFMIVFRVLCGEWIESIWW
M.d CIIIFIFAVMGMLFGKNYIDHKDRFKDHELPRWNFTDFMHSFMIVFRVLCGEWIESMWD

IIS6

1014

1.1 CMWIASKVCVPPFLATTVIGNLVVL-----NLFALLLSFGASNLSAAGGNDEDTNKL
1.2 CMYVAGPTCVPYFLATVLVANLVIL-----NLFALLLSFSMDGSGEEEE-DGKPKDM
1.3 VKGIVGTVR--FFKNGI-VRLLRGXGXS R PGDTDIALEVTEEKDFQG--ENG-DLKNRGL
M.d CMYVGDVSCIPFFLATTVIGNLVVL-----NLFALLLSNFGSSLSAPT-ADNDTNKI

SEAFNRLRLRVFLQKLSIRGLKYVKDKIILCFRQIFNARREALMRQNR--ASTEINQEH

1.1 SEAFNRLRLRVFLQKLSIRGLKYVKDKIILCFRQIFNARREALMRQNR--ASTEINQEH
1.2 SIAVNRFRK-----
1.3 KVEINHIGIPL-----G-
M.d AEAFNRIRARFKNWVKRNIADCFKLIRNKLT-----NQISDQPSHGDNELELGH

RTL-----KKDQHIVSYDVKIGDGMIAIQENGIDDKWQTAIRH-HSL-----

1.1 RTL-----KKDQHIVSYDVKIGDGMIAIQENGIDDKWQTAIRH-HSL-----
1.2 -----LFSFTKRRIKEELFDRIX---RK-----I--AH-SKS---
1.3 -----NESSDPNFPDSSIGEGMDITIQGVNHLNKNKREDXASLCSHG-SKA---
M.d DEIMDGLIKKGKMGKETQLEVAIGDGMIEFTIHGDMKNNPKKSKFINNTMIGNSINHQD

---IKLKNFDDF-----SFDNTSETSYRSHKNRNFNTGSHKGSVVSISNPKEAIILEQP

1.1 ---IKLKNFDDF-----SFDNTSETSYRSHKNRNFNTGSHKGSVVSISNPKEAIILEQP
1.2 KPLIPEANGNGFSHKT-----N-----GYIGGHNNV-PS--E
1.3 LQDFPEDDKLGYSDMSIGRMDISITGEGL--SK-----EWIGEGEEA-VV--E
M.d NRLEHELHNRHGL---SIQDDTASINSYGSHKNRPFKDESHKSAETIEGEEKRDVSKED

NGGVAGHRVLLDSDSFEDEEDYDYDEDEYAKLE-KEIFDATAQEILMHEYPSECCPKDF

1.1 NGGVAGHRVLLDSDSFEDEEDYDYDEDEYAKLE-KEIFDATAQEILMHEYPSECCPKDF
1.2 TQ---TY--IPI-----DQOSDKFAPQTKD-HEEIIYDPLETLEVEDCCPPFV
1.3 NGGKMED--VAM---NNN--NNSYTEDPVIVPNGKS-MEEPVDVDDVVVECCPAIC
M.d -----LGLDEELDEEA-----EGDEGQLDGDII IHAQNDEIIDDYPADCFPDSY

IIIS1

1.1 YLRPLFLAGDPDPSFFWQGWGNLRIKTRFLIENKYFETAVIVMILISSLALALEDVYLP SR
1.2 YRIIP--LGDQHSFFWEAWTQORLMAMRLIENEYFEGVVLGLLILSSFVMTLEDIWFETR
1.3 YRVCPCICGDPDPSFFWQLWYRHRRLQVSRLEENKYFEGVVLTLILSSFVMTLEDIWFETR
M.d YKFPILAGDEDSFFWQGWGNLRLKTFQLIENKYFETAVITMILMSSLALALEDVHLPDR

IIIS2

IIIS3

1.1 PVLQDVLYYMDRIFTVIFFLEMVCKWLALGFVKYFTNAWCWLD FVIVMFSILNLVASF FG
1.2 PVLIDCLYLDRLTIVVFFLETLLKLFAMGPMYFTNAWCWLD FVIVAXSLINFIASLVG
1.3 PLLVDMLYLDRLTIVVFFLETLLKLFAMGCVMYFGNAWCWLD FVIVAVSLINFGASLVG
M.d PVMQDILYYMDRIFTVIFFLEMLIKWLALGFVKYFTNAWCWLD FVIVMLSLINLVAVWSG

IIIS4

1410

IIIS5

1.1 VGNIPIFKTI RTLRGLRPLRALSRIGGMKVVVNA LVQAIPSFNVLLVCLIFWLI FAIMG
1.2 AVNIPIFKTMRTLRALRPLRAMSKMEGMKVVVNA LVGALPSIFNVLLVCLIFWLI FAIIG
1.3 LGNIPIFKTMRTLRALRPLRAMAKMEGMKVVVNA LVGALPSIFNVLLVCLIFWLI FAIIG
M.d LNDIAVFRSMRTLRALRPLRAVSRWEGMKVVVNA LVQAIPSFNVLLVCLIFWLI FAIMG

1494

VQMFAGKYYKCEDAE-GNRLNASYTPDKETCLE-NQYVWNSVHNFNDVGSAYLSLFOVA

1.1 VQMFAGKYYKCEDAE-GNRLNASYTPDKETCLE-NQYVWNSVHNFNDVGSAYLSLFOVA
1.2 VNLFMGT FHKCIDSIXGHKFSHEIIPNKTVCLNQ TNAQWINSKVTFDNVIMAYLALQVA
1.3 INTFMGKFKCLDEETGEKFSHEIIPNKTVCLNETGAVWTNARVNFNDVFIAYLALFOVA
M.d VQLFAGKYFKCKDGN-DTVLSHEIIPNRNACKSE-NYTWENSAMNFDHVGNAYLCLFOVA

Figure 3

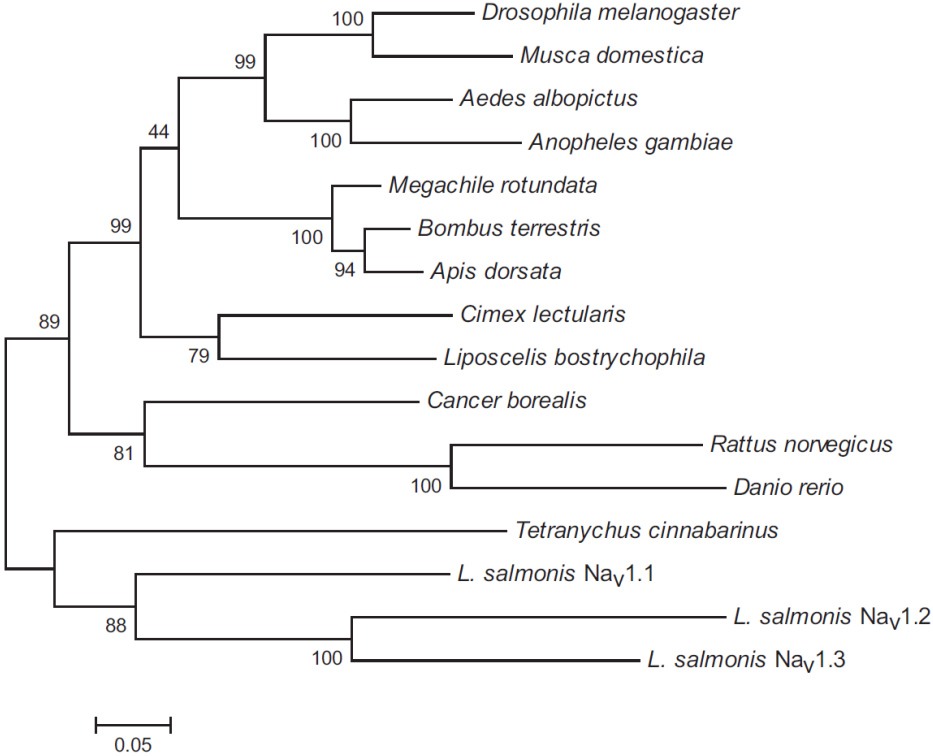


Figure 4

