Deltamethrin resistance in salmon lice: Genetic markers and molecular mechanisms

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Declaration

I, the undersigned, hereby declare that this thesis has been composed entirely by me and has not been submitted for any other degree. The work presented in this thesis, except where specifically acknowledged, is the result of my own investigations.

Word count: Approximately 65,000 words

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October 2021

This is to certify that this thesis for the degree of Doctor of Philosophy entitled “Deltamethrin resistance in salmon lice: Genetic markers and molecular mechanisms” submitted to the University of Stirling (UK), is an original work carried out by Claudia Tschesche under our supervision.

______________________ ______________________

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- Greta Carmona Antoñanzas, Kari O. Helgesen, Joseph L. Humble, Claudia Tschesche, Marit J. Bakke, Louise Gamble, Michaël Bekaert, David I. Bassett, Tor E Horsberg, James E. Bron, and Armin Sturm (2018) Mutations in voltage-gated sodium channels from pyrethroid resistant salmon lice (*Lepeophtheirus salmonis*). Pest Management Science 75:527-536.
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Book chapters

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- **International Sea Lice Conference, 4-8th November 2018, Punta Arenas, Chile**

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  Oral presentation 'New insights into AMX® (deltamethrin) resistance in salmon lice'

- **Fish Vet Society Annual Conference, 20th-21st May 2018, Edinburgh, GB**

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List of abbreviations

ABC transporter	Adenosine triphosphate binding cassette transporter
ace1a	Acetylcholinesterase 1a
ace1b	Acetylcholinesterase 1b
AChE	Acetylcholinesterase
Ala	Alanine
Asp	Aspartic acid
ATP	Adenosine triphosphate
AWERB	Animal Welfare Research Body
Bax	B-cell lymphoma 2-associated X protein
Bcl-2	B-cell lymphoma 2
BLAST	Basic Local Alignment Search Tool
Ca^{2+}	Calcium ion
CaE	Carboxylesterase
cDNA	Complementary DNA
Cl^{-}	Chloride Ion
CLC	Voltage-gated chloride channels
Ct value	Cycle threshold value
CYP	Cytochrome P450 monooxygenase
Cys	Cysteine
CYTB	Cytochrome B
cytC	Cytochrome C
COX	Cytochrome c oxidase
DA	deltamethric acid (3-(2,2-Dibromovinyl)-2,2-dimethyl-cyclopropanecarboxylic acid)
DmAChE	*Drosophila melanogaster* acetylcholinesterase
$DmNa_v$	*Drosophila melanogaster* voltage-gated sodium channel
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBI	European Bioinformatics Institute

EC_{50}	Median effective concentrations
ECT	Electron transport chain
ENA	European Nucleotide Archive
ETC	Electron transport chain
F1	First filial generation
F2	Second filial generation
FRET probe	Fluorescence energy transfer probes
GABA	Gamma-Aminobutyric acid
$GABA_A$ receptor	GABA-type A receptor
Glu	Glutamic acid
GSH	Glutathione
GST	Glutathione-S-transferase
His	Histidine
Ile	Isoleucine
K^+	Potassium Ion
Kdr	Knockdown resistance
Met	Methionine
miRNA	Micro RNA
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger RNA
mtDNA	Mitochondrial genome
Na^+	Sodium ion
Na_v	Voltage-gated sodium channel
Na_v1	Pore-forming α-subunit of Na_v, which is functional on its own
NCBI	National Center for Biotechnology Information
ND	NADH dehydrogenase
3-PBA	3-phenoxybenzoic acid
PCR	Polymerase chain reaction
PEG_{300}	Polyethylene glycol, $M_n = 300$
P-gp	P-glycoprotein

Phe	Phenylalanine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
Ser	Serine
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
UoS	University of Stirling
UTR	Untranslated regions
Val	Valine
VGCC	Voltage-gated calcium channels
WT	Wild type

Abstract

Resistance of salmon lice (*Lepeophtheirus salmonis*) against the pyrethroid deltamethrin used in bath treatments is common, but the mechanisms underlying resistance are unknown. In insects, deltamethrin resistance can involve mutations of voltage-gated sodium channels (Na_v) considered the target-site of pyrethroids as well as metabolic detoxification through carboxylesterases (CaEs). In *L. salmonis*, deltamethrin resistance is mainly inherited maternally and associated with single nucleotide polymorphisms (SNPs) in the mitochondrial genome (mtDNA). In addition, a potential target-site mutation (I936V) has been identified in the *L. salmonis* Na_v homologue $LsNa_v1.3$.

This PhD thesis investigated the relative contribution of Na_v target-site mutations and mtDNA mutations in deltamethrin resistance in *L. salmonis. L. salmonis* from farm sites were rated as deltamethrin resistant or susceptible in bioassays and genotyped for the $LsNa_v1.3$ mutation I936V and previously identified resistance-associated mitochondrial SNPs. The results provided no evidence for a role of I936V in deltamethrin resistance, while confirming its association with several mtDNA SNPs. This conclusion was further supported by the genotyping of deltamethrin resistant and susceptible *L. salmonis* derived from a crossing experiment. Further experiment assessed the association of mitochondrial SNPs with deltamethrin resistance. In *L. salmonis* from farm sites, several mitochondrial haplotypes associated with deltamethrin resistance were identified, suggesting that deltamethrin resistance evolved at least two times independently. The association of a previously unknown haplotype with deltamethrin resistance was demonstrated in toxicity and genetic studies that support the hypothesis that SNP T8600C in cytochrome c oxidase subunit 1 (Leu107Ser) is related to the resistance mechanism.

Finally, potential roles of the CaE gene family in deltamethrin resistance were examined. *L. salmonis* CaE sequences were identified and annotated, and CaE sequences predicted to be catalytically competent were studied regarding their transcript and SNP expression in resistant and susceptible lice. Results suggested that CaEs are not major determinants of deltamethrin resistance.

Confirmation of ethical approval

All research projects involving the University of Stirling (UoS) are subject to a thorough Ethical Review Process prior to any work being approved. The experiments within the present PhD project were assessed by the UoS Animal Welfare Ethical Review Body (AWERB) and passed the ethical review process. Laboratory infections of Atlantic salmon with *L. salmonis* were performed under a valid UK Home Office licence and at low parasite densities unlikely to compromise fish welfare.

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Chapter 1: General introduction

1.1 Background

Aquaculture is the world's fastest growing animal production sector with an annual growth rate of 5.3% between 2001 and 2018 (FAO, 2020). In 2018, aquaculture accounted for 46% of the total fish production (FAO, 2020). The demand for cultured fish has steadily grown. Since 2013, salmonids are the largest share by value in world trade (FAO, 2018). Today, the most produced salmonid species is Atlantic salmon (*Salmo salar*), followed by rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*). In 2018, 2.44 million tonnes of farmed Atlantic salmon were produced globally, mainly in Norway (53% global share), Chile (27% global share), Scotland (7% global share), and Canada (5% global share) (FAO, 2021).

During production in offshore net cages, salmon are in constant contact with their surrounding environment. Pathogens, including bacteria, viruses, and parasites can therefore spread through the seawater between farmed and wild fish, as well as among cages within and between farms (Johansen et al., 2011; Tengs and Rimstad, 2017). Transmission of pathogens is favoured by a year-round high population density in fish farms (Johansen et al., 2011).
Outbreaks of bacterial diseases in salmonid aquaculture have strongly decreased since the late 1980s following the development of vaccines. Atlantic salmon vaccines have also been developed against viral diseases (reviewed by Ma et al., 2019), but some common viral diseases are not yet covered (Garseth et al., 2018; Wessel et al., 2018). Viral diseases still pose a threat to aquaculture due to the short generation times, high mutation rates, and large population sizes of viruses (Walker and Winton, 2010). The most prevalent parasitic diseases in salmonid aquaculture are amoebic gill disease caused by *Neoparamoeba perurans* (Munday et al., 2001), and infestations by sea lice (Copepoda, family Caligidae), i.e., *Lepeophtheirus salmonis* in the northern hemisphere and *Caligus rogercresseyi* in the southern hemisphere (Aaen et al., 2015; Torrissen et al., 2013).

Sea lice are ectoparasites infecting farmed and wild marine fish, feeding on the mucus, skin, and blood of the host (Boxaspen, 2006). Depending on the severity of infection, sea lice can cause adverse effects in their fish hosts that include skin lesions, which are associated with a high risk of secondary infections, as well as osmoregulatory dysfunction, immunosuppression, increased stress, and reduced food conversion and growth rates (Grimnes and Jakobsen, 1996; Wootten et al., 1982). In 2018, the estimated global cost of sea lice infections to the salmon industry were approximately USD $873 million / GBP £700 million (Brooker et al., 2018b), comprising mainly the cost of treatments and to a lesser extent loss in production.

During salmon production, sea lice infestations are controlled by integrated pest management strategies combining non-medicinal approaches (reviewed by Brooker et al., 2018a; Holan et al., 2017) and chemical treatments (reviewed by Overton et al., 2019). Pharmaceuticals used for salmon delousing agents are administered as medicated feeds or topical bath treatments. Depending on the country, oral treatments include the avermectin emamectin benzoate and some benzoylureas, while bath treatments include the organophosphate azamethiphos, the disinfectant hydrogen peroxide, the neonicotinoid imidacloprid, and the pyrethroids cypermethrin and deltamethrin (Helgesen et al., 2020).

Deltamethrin (AMX®, PHARMAQ AS) is one of the approved salmon delousing drugs in Norway, Chile, Scotland, the Faroe Islands, and Ireland. However, as with most available sea louse treatments, losses in efficacy have been reported (Fjørtoft et al., 2020, 2017; Helgesen et al., 2020). At present, the molecular mechanisms of deltamethrin resistance in sea lice are largely unknown.

This introductory chapter provides a brief overview of the biology of sea lice and details on methods to control sea lice infestations within farmed fish. The focus is on the pyrethroid deltamethrin, one of the approved chemical salmon louse treatments in Norway, Chile, Scotland, the Faroe Islands, and Ireland. This chapter will summarise findings on the mode of action of deltamethrin and the mechanisms of deltamethrin resistance in both terrestrial arthropods and sea lice.

1.2 Biology of sea lice

1.2.1 Classification and geographical distribution

Sea lice are marine ectoparasitic copepods of the family Caligidae (Burmeister, 1835; Copepoda; Siphonostomatoida). They infest wild and farmed salmonid fish in the North Atlantic, Pacific Ocean, and adjacent seas. In the Southern hemisphere, the Pacific coast of southern Chile, and the Atlantic coast of Argentina, *C. rogercresseyi* is the most prevalent sea louse species (Johnson and Jakob, 2012). In the Northern hemisphere, the Pacific coast of Canada, Korea, Japan and Russia, and the Atlantic coast of Northern United States, Norway, Ireland, and Scotland *L. salmonis* is most frequently occurring, also referred to as salmon louse (Johnson and Jakob, 2012).

L. salmonis from the Pacific and Atlantic Ocean are reproductively compatible, but genetic studies provide strong evidence for two different subspecies, *L. salmonis salmonis* in the Atlantic Ocean and *L. salmonis oncorhynchi* in the Pacific Ocean (Skern-Mauritzen et al., 2014; Todd et al., 2004; Yazawa et al., 2008). Two extensive studies based on large sets of microsatellite markers and single nucleotide polymorphisms (SNPs) found very little evidence for

genetic differentiation among *L. salmonis salmonis* (Besnier et al., 2014; Glover et al., 2011). Most likely, the low degree of genetic differentiation in Atlantic *L. salmonis* results from long distance migration of Atlantic salmon originating from different freshwater systems, which mix in offshore feeding grounds (Hansen and Jacobsen, 2003). Salmon lice infest seawards migrating smolts in coastal zones and are transported to feeding grounds where cross-infection may occur (Berland, 1993; Holst et al., 1993; Jacobsen and Gaard, 1997). Further contributing to the genetic exchange between *L. salmonis* populations at a more local scale is the parasite's planktonic dispersal at nauplii and copepodid larval stages, which has been estimated to allow for transmission up to 45 km (Johnsen et al., 2016; Salama et al., 2013). Similarly, in the Pacific Ocean, little population genetic differentiation was found among *L. salmonis oncorhynchi* (Messmer et al., 2011).

1.2.2 Life history of *L. salmonis*

Adult *L. salmonis* exhibit sexual dimorphism. Females (8-11 mm) are larger than males (5-6 mm), have a more prominent genital segment, and extrude paired egg strings (12-20 mm) (Johnson and Albright, 1991a; Schram, 1993). The average number of eggs per string ranges from 123 to 183 eggs at 7.2˚C (Heuch et al., 2000).

L. salmonis has a direct life cycle divided into the free-living planktonic phase with two nauplii and one copepodid stage, the host-attached phase with two chalimus stages, and the mobile host-associated phase with two preadult and one adult stage (Hamre et al., 2013). Gravid females produce egg strings and the lecithotrophic planktonic nauplii hatch directly into the water column. Nauplii moult through two stages before reaching the infective copepodid stage (Johnson and Albright, 1991a). Copepodids must settle on a host fish to survive and progress to the next stage. Physical and chemical cues are important for host detection. They involve factors that increase the orientation towards the natural environment of the host (Bricknell et al., 2006; Heuch et al., 1995; Tucker et al., 2000), as well as factors that cause recognition of a potential host (Heuch et al., 2007; Heuch and Karlsen, 1997; Hevrøy et al., 2003; Komisarczuk et al., 2017; MacKinnon, 1998; Mordue and Birkett, 2009). Copepodids moult into chalimus stages I and II (Hamre et al., 2013), which are both attach to the host via a frontal filament extension of the cuticle (Bron et al., 1991). This filament is inserted through the host's epidermis and anchored with an adhesive basal plate between the epidermis and the underlying basement membrane. Chalimus II moults to the first preadult stage. Preadults I and II as well as adults are mobile (Johnson and Albright, 1991a; Ritchie et al., 1996a; Schram, 1993). They move over the surface of the host but can attach by using their cephalothorax as a suction pump (Kabata, 1982). For reproduction, male adults with female adults, but preferably with preadult II females, form a

precopulatory complex and mate. Females store the received sperm and can fertilise up to eleven clutches of eggs (Heuch et al., 2000; Ritchie et al., 1996b). Among females, polyandry exists (Todd et al., 2005).

The development of *L. salmonis* is temperature and salinity dependent (Johnson and Albright, 1991b; Samsing et al., 2016). The generation time of salmon lice has been estimated to range between 50 days at 12°C and 114 days at 7°C (Tully, 1992). Nauplii do not develop to the infective copepodid stage below salinities of 25‰ (Johnson and Albright, 1991b).

1.2.3 Sea lice-host interaction and pathogenicity

L. salmonis requires salmonid host fish to complete its life cycle (Johnson and Albright, 1991a). While the Atlantic salmon louse *L. salmonis salmonis* infests Atlantic salmon (Wootten et al., 1982), Arctic charr (*Salvelinus alpinus*) (Finstad et al., 1995) and farmed rainbow trout (Jackson et al., 1997), the Pacific salmon louse *L. salmonis oncorhynchi* infests rainbow trout (Jackson et al., 1997), sea trout (*Salmo trutta*) (Tingley et al., 1997), brook trout (*Salvelinus fontinalis*), pink salmon (*Oncorhynchus gorbuscha*), coho salmon, sockeye salmon (*Oncorhynchus nerka*), chum salmon (*Oncorhynchus keta)*, Chinook salmon (*Oncorhynchus tshawytscha*) (Nagasawa, 1987), and farmed Atlantic salmon.

Preadult and adult salmon lice graze on mucus, skin, and underlying tissue of their hosts using their rasping mouthparts (Brandal, 1976; Kabata, 1974; White, 1942). Sea lice grazing can induce increased mucus discharge, changed mucus biochemistry, epithelium loss, bleeding, and tissue necrosis (Firth et al., 2000; Grimnes and Jakobsen, 1996). Highly infested host fish show impaired osmoregulatory and respiratory abilities, reduced appetite, and reduced food-conversion efficiency and growth (Bowers et al., 2000; Grimnes and Jakobsen, 1996). These factors weaken and stress the host, which may result in lower swimming speed, impaired immunocompetence, increased susceptibility to secondary infections and possibly death (Finstad et al., 2000; Mustafa et al., 2000). Experimental studies have shown that salmon louse infections at levels corresponding to densities of 0.1 adult lice g^{-1} fish alter the host's physiology and more than 0.75 chalimus larvae g^{-1} fish can cause death (Grimnes and Jakobsen, 1996; Wagner et al., 2008).

1.3 Sea lice management at aquaculture sites

1.3.1 Non-medicinal control methods

During salmon production, sea lice infestations are controlled by non-medicinal methods (reviewed by Brooker et al., 2018a; Holan et al., 2017) and chemical treatments (reviewed by Overton et al., 2019). During the last decade different non-medicinal sea louse control approaches have been widely implemented at an industrial scale (Jensen et al., 2020). Since 2016, the most frequently applied technical non-medicinal control method is thermal delousing (Fig. 1.1), which is based on the inactivation of lice after short exposure to warm water (up to 36°C for approximately 30 s; Thermolicer®, steinsvik.no; Optilice®, optimar.no) (Grøntvedt et al., 2015; Roth, 2016). The second most used non-medicinal treatment tool is mechanical removal of lice, which involves removal of lice by soft brushes and/or high-pressure pumps (FSL delouser, fls.no; SkaMik, moenmarin.no; Hydrolicer®, smir.no) (reviewed by Overton et al., 2019). In addition, freshwater treatments are widely used to reduce sea lice infestations (Hjeltnes et al., 2017; Powell et al., 2015). Treatments are usually performed in well boats and involve 5 to 8 h exposure to freshwater (reviewed by Groner et al., 2019).

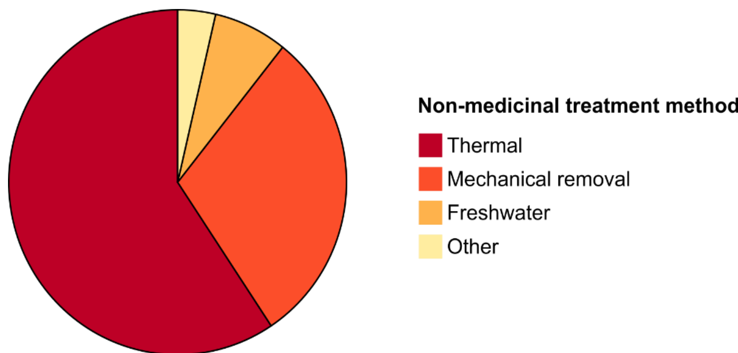

Figure 1.1 Proportion of non-medicinal treatments of salmon lice in Norway based on the number of weeks in 2019. Non-medicinal treatments have been reported by Norwegian fish farms in weekly mandatory salmon lice reports to the Norwegian Food Safety Authority. Non-medicinal treatments were subdivided into: Freshwater, freshwater bath treatments; Mechanical removal, treatments involving water pressure or brushes; Thermal, treatments using temperate water (Helgesen et al., 2020).

Besides these intervention methods, a variety of non-medicinal preventive tools are available to control sea lice. A common non-medicinal approach is the use of cleaner fish, which are stocked into fish cages and prey upon sea lice attached to fish (Brooker et al., 2018a). For this purpose, two species are currently being farmed in the North Atlantic, ballan wrasse (*Labrus bergylta*) (Leclercq et al., 2014) and lumpsucker (*Cyclopterus lumpus*) (Imsland et al., 2014; Powell et al., 2017). In addition, wild-caught wrasse species including goldsinny wrasse (*Ctenolabrus rupestris*), corkwing wrasse (*Symphodus melops*), and rockcook wrasse (*Centrolabrus exoletus*) are stocked into fish cages to feed on salmon lice (Directorate of Fisheries, 2020). Other approaches to prevent sea lice infestations involve barrier technologies such as snorkel cages and net skirts

(Stien et al., 2016; Tveit, 2012). Spatial overlap between host fish and salmon lice can be reduced by promoting deep swimming behaviour of fish via by deep feeding and lighting (Bui et al., 2020; Frenzl et al., 2014; Hevrøy et al., 2003). Moreover, commercially available functional health feeds aim at reducing the attachment success of sea lice or facilitating effective immune responses of host fish against lice infestations (Jensen et al., 2015; Martin and Król, 2017). Selective breeding for sea lice resistance can also be used to increase resistance of fish against sea lice (Gharbi et al., 2015; Holborn et al., 2019). In addition, the development of effective vaccines against sea lice remains key target (Contreras et al., 2020; Swain et al., 2018).

1.3.2 Chemical treatments

From the late 1970s until approximately 2015, sea louse control relied to a large extent on the use of chemical salmon delousing agents (Jensen et al., 2020). Since 2017, the number of chemical treatments has been overtaken by the number of treatments using non-medicinal approaches (Fig. 1.2). Nonetheless, drug treatments are still widely used to control of sea lice infestations.

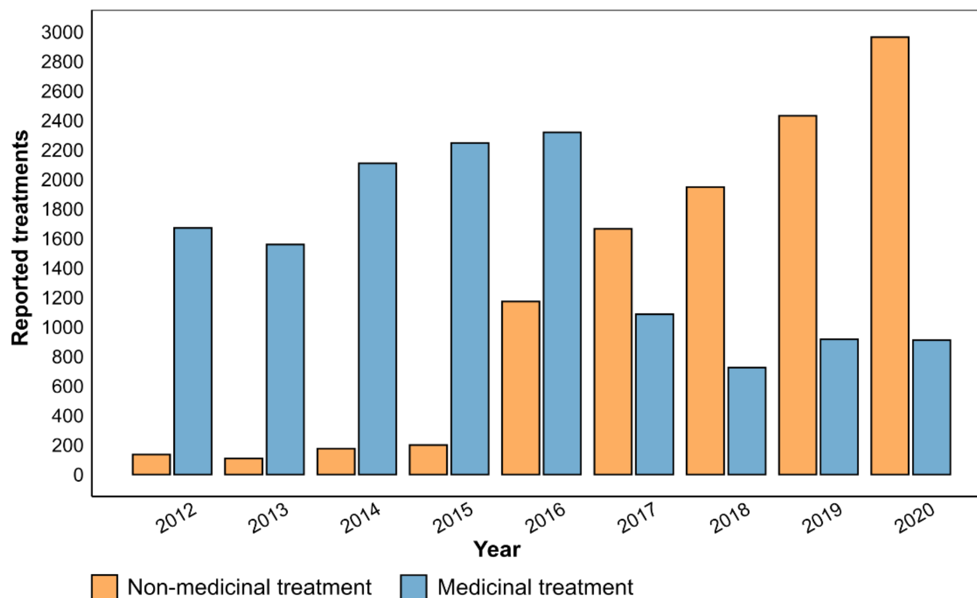

Figure 1.2 Number of non-medicinal and medicinal treatments of salmon lice in Norway between 2012 and 2019. Treatments have been reported by Norwegian fish farms in weekly mandatory salmon lice reports to the Norwegian Food Safety Authority. Records on non-medicinal and medicinal treatments in Norway are available from Norwegian fish health Barentswatch 2020 (https://www.barentswatch.no/en/fishhealth/).

Currently, five groups of medicinal compounds are used to treat sea lice infestations: organophosphates, disinfectants (hydrogen peroxide), avermectins, benzoylureas, and pyrethroids. In Scotland, four products hold a market authorisation. The organophosphate azamethiphos (Salmosan Vet®, Fish Vet Group (rebranded to PHARMAQ Analytiq)); hydrogen peroxide (Salartect 500® or 350®, Brenntag UK Ltd; Paramove 35® or 50®, Solvay

Interox); the avermectin emamectin benzoate (Slice®, MSD Animal health), and the pyrethroid deltamethrin (AMX®, PHARMAQ AS). In Norway, the benzoylureas diflubenzuron (Lepsidon®, EWOS) and teflubenzuron (Ektobann®, Skretting) are additionally authorised, and in July 2021 the neonicotinoid imidacloprid (Ectosan Vet, BMK08, Benchmark) received market authorisation from the Norwegian Medicines Agency in conjunction with a water filtration system (CleanTreat®, Benchmark). In Chile, the benzoylureas lunfenuron (Imvixa®, Elanco) and hexaflumeron (Alpha Flux®, PHARMAQ AS) are also used.

Pharmaceuticals used for salmon delousing agents are administered as medicated feeds or topical bath treatments. Oral treatments include emamectin benzoate, diflubenzuron, teflubenzuron, and lufenuron, while bath treatments include azamethiphos, hydrogen peroxide, cypermethrin, deltamethrin, imidacloprid, and hexaflumuron (Helgesen et al., 2019). Bath treatments are performed in well boats, or in the sea-cages in a raised net enclosed with a tarpaulin while fish are oxygenated (PHARMAQ AS, 2017).

Organophosphates: Organophosphorus compounds were the first drugs used to treat sea lice infestations, with metrifonate being introduced in 1980 in Norway, followed by dichlorvos in 1986 (Grave et al., 2004; Torrissen et al., 2013). In 1994, dichlorvos was substituted by azamethiphos due to higher delousing rates, higher safety margins, and lower residues in the environment (Denholm et al., 2002; Rae, 2002; Roth et al., 1996). While azamethiphos use in Norway stopped after 1999 due to resistance problems, it was reintroduced to the market in 2008 as alternative treatments became inefficient (Torrissen et al., 2013).

In Norway, it was the most widely used chemical salmon delousing agent between 2010 and 2012 (Fig. 1.3) (Norwegian Institute of Public Health, 2021). Based on sales (in kg) in Scotland, azamethiphos had a marked share of 54% in 2008, which increased to 87% in 2017 (Fig. 1.4).

Hydrogen peroxide: Hydrogen peroxide, an oxidative agent widely used as a disinfectant, was introduced as a salmon delousing bath treatment from 1993 in Norway and Scotland, compensating for the decreasing efficacy of azamethiphos (Denholm et al., 2002). However, as hydrogen peroxide possesses a narrow therapeutic index and its dosage requires careful consideration of environmental temperature, the usage of hydrogen peroxide almost discontinued with the introduction of new delousing drugs with higher safety margins in 1997 in Norway and in 1999 in Scotland (Helgesen et al., 2015). Ten years later, hydrogen peroxide was reintroduced as resistance levels towards other salmon delousing agents increased (Aaen et al., 2015).

Pyrethroids: Pyrethrum, an extract from chrysanthemum flowers containing pyrethrins, was introduced as a bath treatment in 1989. During the mid-1990s, it was replaced by the chemically

related synthetic pyrethroids cypermethrin and deltamethrin, which show a superior efficacy and therapeutic index (Denholm et al., 2002; Roth et al., 1993; Torrissen et al., 2013). Cypermethrin was introduced first (1996 in Norway, 1997 in Scotland), followed by deltamethrin (1998 in Norway, 2008 in Scotland) (Denholm et al., 2002; Grave et al., 2004).

Based on sales (in kg) in Norway in 2002, pyrethroids had a market share of 81% (excluding hydrogen peroxide). While deltamethrin had its largest market share of 16 to 22% (excluding hydrogen peroxide) between 2002 and 2008, its market share is below 2% since 2009. The number of deltamethrin prescriptions for salmon delousing in Norway decreased from 1155 in 2012 to 73 in 2019 (Helgesen et al., 2020). Nonetheless, the largest total amount of deltamethrin was used in 2014 (158 kg) (Fig. 1.5), the year with the highest consumption of salmon delousing agents in Norway (12812 kg, excluding hydrogen peroxide). In Scotland, the largest absolute amount of deltamethrin was used in 2012 (21 kg, 4% of all chemical salmon delousing agents excluding hydrogen) (Fig. 1.4 and 1.5). Since 2017, its market share is below 1 %.

Avermectins: Emamectin benzoate is used in Norway since 1999 and in Scotland since 2000 (Aaen et al., 2015; Grave et al., 2004). In Norway, emamectin benzoate was the most widely used delousing agent between 2005 and 2008 (Fig. 1.3). Since then, its market share based on sales (in kg) is below 3.2% (excluding hydrogen peroxide; Fig. 1.4). In Scotland in 2005, emamectin benzoate accounted for 86% of all chemical treatments. However, in 2017 its marked share decreased to 12%.

Benzoylureas: Benzoylureas were introduced in Norway in 1996 and in Scotland in 2007 (Aaen et al., 2015). However, since 2013, the benzoylurea teflubenzuron is no longer used in Scotland (Fig. 1.4). In contrast, teflubenzuron and diflubenzuron are still used in Norway. Since 2013, diflubenzuron is the most widely used chemical delousing agent in Norway. In 2019, its market share based on sales (in kg) was 73.8% (excluding hydrogen peroxide; Fig. 1.3).

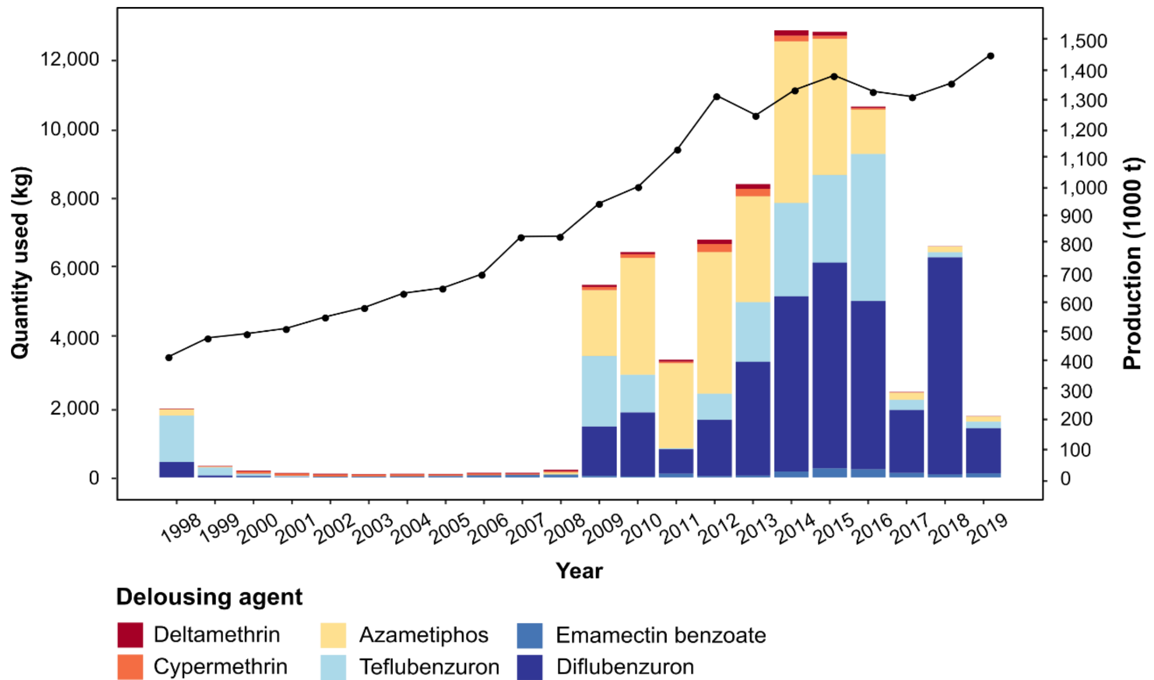

Figure 1.3 Estimated use of different salmon delousing compounds based on sales (in kg) in Norway from 1998 to 2019. The secondary y-axis shows the total annual product (1000 t) of salmonids in Norway. Records on the utilisation of salmon delousing agents in Norway is available from the Norwegian Institute of Public Health (https://www.fhi.no/hn/legemiddelbruk/fisk/2019-bruk-av-legemidler-i-fiskeoppdrett/). Records on the annual production (biomass harvested) of Atlantic salmon, rainbow trout, and arctic char are available from Statistics Norway (https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett/aar-forelopige).

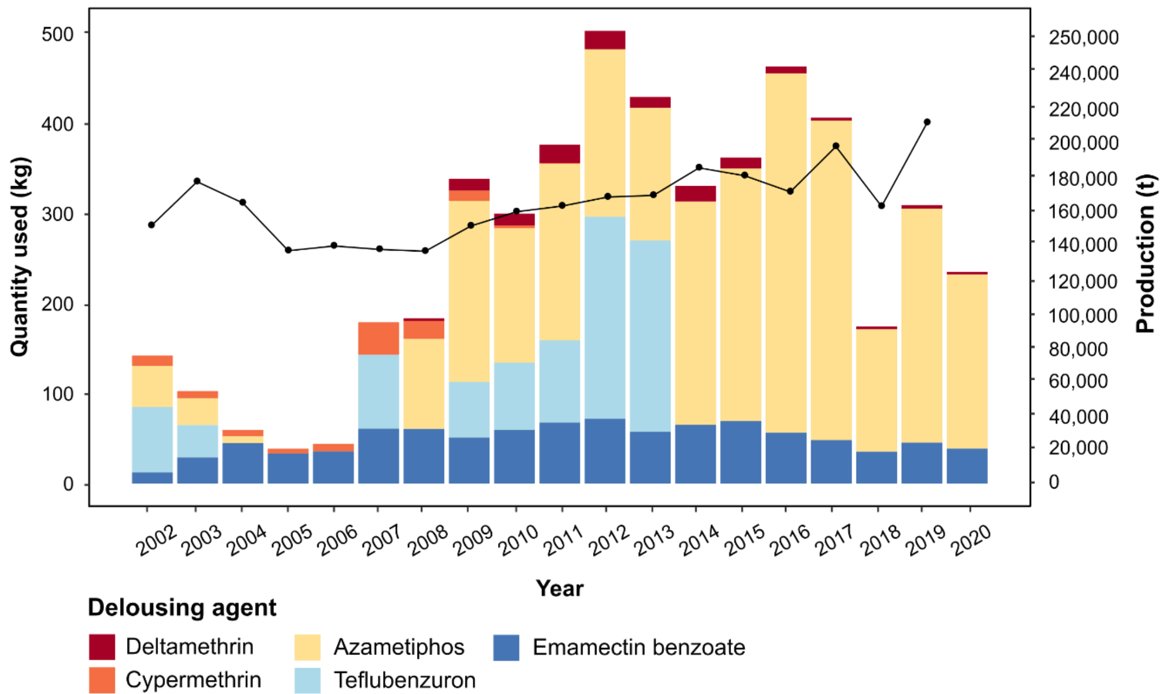

Figure 1.4 Estimated use of different salmon delousing compounds based on sales (in kg) in Scotland from 2002 to 2020. The secondary y-axis shows the total annual product (t) of salmonids in Scotland. Records on the utilisation of salmon delousing agents in Scotland is available from the Scottish Environment Protection Agency (http://aquaculture.scotland.gov.uk/). Records on the annual production of Atlantic salmon and rainbow trout are available from Marine Scotland (https://data.marine.gov.scot/dataset/scottish-fish-farm-production-survey-data).

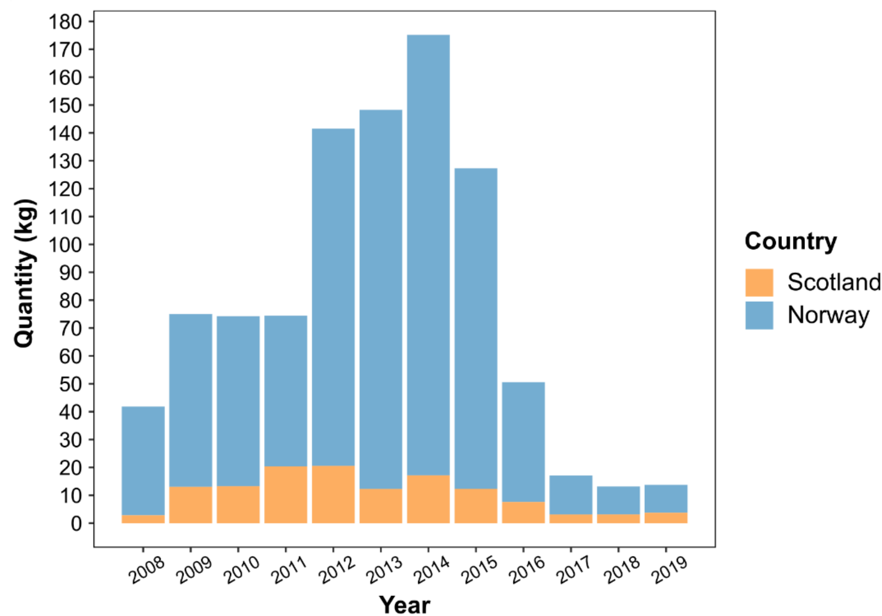

Figure 1.5 Estimated use of deltamethrin compounds based on sales (in kg) in Scotland and Norway from 2008 to 2019. Records on the utilisation of deltamethrin in Scotland is available from the Scottish Environment Protection Agency (http://aquaculture.scotland.gov.uk/). Records on the use of deltamethrin in Norway is available from the Norwegian Institute of Public Health (https://www.fhi.no/hn/legemiddelbruk/fisk/2019-bruk-av-legemidler-i-fiskeoppdrett/).

1.3.3 Integrated pest management

Integrated pest management (IPM) for sea lice control involves a multifaceted and systematic approach, which combines a variety of tools for most effective control. Key elements are (I) Monitoring, (II) husbandry and management, (III) prevention, and (IV) intervention and resistance monitoring (Brooks, 2009).

(I) Monitoring: To date, most sea lice management plans rely on monitoring of lice levels and implementation of treatment methods as soon as national thresholds/limits are exceeded. In Norway, fish farmers must report the number of sea lice on fish on a weekly basis. The maximum permitted average number of adult female lice per fish is 0.5 and in certain regions during spring 0.2 (Norwegian Ministry of Trade, Industry and Fisheries, 2012). In Scotland weekly sea lice counts exceeding an average of two adult female lice per fish must be reported and result in an increased monitoring by the Fish Health Inspectorate. An average of six adult female lice per fish requires the implementation of enforcements actions (Marine Scotland, 2019). In addition, effective salmon louse management can benefit from epidemiological modelling (Groner et al., 2016).

(II) Husbandry and management: Sea lice infestations can be controlled by management plans involving coordinated stocking of single-year classes, fallowing of farm sites between production cycles, and limited production periods (Bron et al., 1993; Brooks, 2009; Werkman et al., 2011). Monitoring of fish health status and frequent removal of moribund fish and runts also supports sea lice control. Moreover, clean nets increase water flow and minimise the retention of sea lice larvae, and prevention of fish escapes reduces the risk of sea lice transfer to other farms and wild fish (Costello, 2004).

(III) Prevention: Sea lice infestations can also be reduced by a variety of non-medicinal preventive tools such as the use of cleaner fish (Brooker et al., 2018a), barrier, deep-feeding/deep-lights, or functional feeds technologies (reviewed by Barrett et al., 2020) (see section 1.3.1).

(IV) Intervention and resistance monitoring: Intervention methods to control sea lice infestations combine non-medicinal treatments, i.e., thermal delousing, mechanical delousing, and freshwater treatment (see section 1.3.1) (reviewed by Holan et al., 2017), and chemical treatments with prescribed medicines (see section 1.3.2) (reviewed by Overton et al., 2019). However, overuse and/or incorrect use of a limit number of non-medicinal and chemical treatments can lead to resistance development (SEARCH Consortium, 2006). It is therefore important to monitor treatment efficacies and rotate treatments with different modes of action when first signs of resistance emerge (Brooks, 2009).

1.4 Status of drug resistance in *L. salmonis*

Organophosphates were the dominating treatment method to control salmon lice infections in the North Atlantic in the 1980s until development of resistance led to the introduction of hydrogen peroxide and pyrethroid in the early 1990s (Denholm et al., 2002; Jones et al., 1992; Roth et al., 1996). However, moderate but clinically relevant resistance of Atlantic *L. salmonis* populations against pyrethroids has already been reported in the mid-2000s (Sevatdal and Horsberg, 2000). Between 2001 to 2003, sensitivity to pyrethroids was investigated in Ireland, Norway, and Scotland, and maximal observed resistance ratios, defined as the quotient of median effective concentrations (EC_{50}) in tested and reference strains, were 11.4 for deltamethrin and 7.0 for cypermethrin (Sevatdal et al., 2005a). Deltamethrin bioassays performed between 2007 and 2012 in Norway showed the emergence of high-level resistance since 2008, characterised by resistance ratios of ≥80 (Jensen et al., 2020; Sevatdal et al., 2005a). Reduced sensitivity to hydrogen peroxide was first reported in Scotland in 2000 (Treasurer et al., 2000) and in Norway the first case report was published in 2015 (Helgesen et al., 2015). As an alternative control method, the avermectin emamectin benzoate was approved in all salmon producing countries in 1999/2000. However, treatment efficacy varied significantly between 2002 and 2006 (Lees et al., 2008), and in 2008 reduced sensitivity to emamectin benzoate resistance was confirmed (Espedal et al., 2013). Nowadays, increasing resistance formation has made emamectin benzoate ineffective against established *L. salmonis* infections in the North Atlantic, but it is still used as a preventative treatment at the beginning of the production cycle.

Comprehensive data on resistance to chemotherapeutants in commercial salmonid aquaculture is only available since 2013 and records are limited to Norway. In that year, the Norwegian Food Safety Authority introduced an annual resistance surveillance programme, which combines active surveillance by bioassays and molecular tests for resistance, as well as passive surveillance by prescriptions of medicines, non-medicinal treatments and reported sensitivity data.

In 2019, low mortalities in bioassays with low concentrations of azamethiphos, emamectin benzoate, and deltamethrin showed widespread reduced sensitivity to these drugs along the Norwegian coast (Fig. 1.6) (Helgesen et al., 2020). Low mortalities in high concentration bioassays further suggested low treatment efficacy in most tested areas. Only hydrogen peroxide yielded higher mortalities in bioassays than the other tested compounds, indicating better treatment results.

Since 2014, the Norwegian Food Safety Authority also applies molecular tests for resistance to pyrethroids, azamethiphos and hydrogen peroxide on lice from farms in the south of Norway. In 2019, molecular testing of 30 lice from three farms showed an average of 62% azamethiphos resistant lice and 59% pyrethroid resistant lice (Helgesen et al., 2020). Spatially and temporally more comprehensive molecular studies by Fjørtoft et al. (2021, 2020, 2019) revealed more widespread resistance to pyrethroids and azamethiphos throughout the North Atlantic. Between 2000 and 2016/17 the authors genotyped ~15000 lice for pyrethroid resistance and ~2000 lice for multi-resistance to pyrethroid and azamethiphos (Fjørtoft et al., 2021, 2020). The studies showed that almost all individuals from North Atlantic areas with intensive aquaculture were genotypically resistant to pyrethroid and azamethiphos. In 2017, the pyrethroid resistance marker was present in >90% and >70% of all tested lice from aquaculture-intensive regions of Norway and Scotland, respectively (Fjørtoft et al., 2020). Multi-resistant lice were found on wild salmonids, as well as on farmed salmonids in areas where delousing agents have not been used (Fjørtoft et al., 2021).

The only compounds against which reports of resistance do not yet exist are benzoylureas.

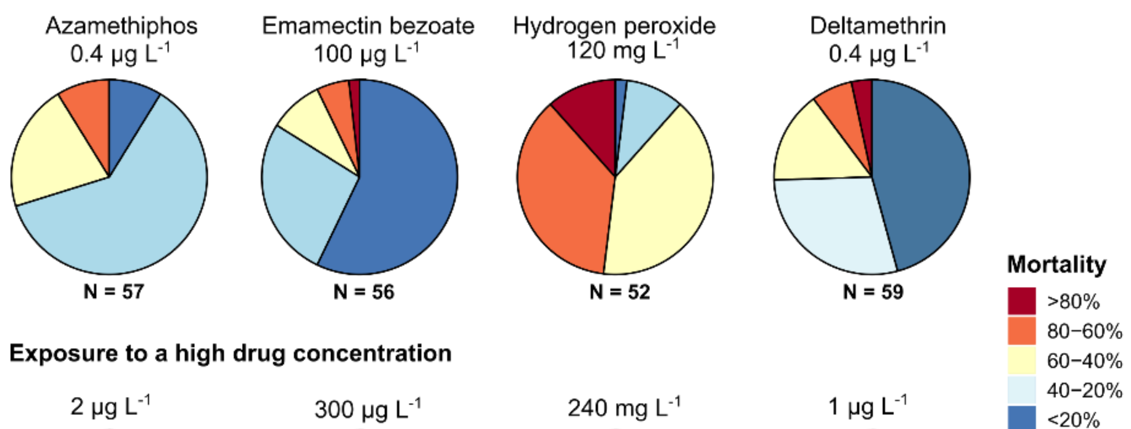

Figure 1.6 Proportions of *L. salmonis* bioassays with different percent mortalities at a low and a high concentration of azamethiphos, emamectin benzoate, hydrogen peroxide, and deltamethrin in 2019. N: Number of tested farms. Records of bioassay data are available from Helgesen et al., 2020.

1.5 Assessment of drug susceptibility in sea lice

1.5.1 Bioassays

The susceptibility of salmon lice towards a pesticide is traditionally determined in bioassays. Standard bioassays are performed to determine the EC_{50}, the toxicant concentration that immobilises half of the exposed lice (SEARCH Consortium, 2006). They are developed for laboratory-reared first-generation parasites or parasites directly collected from the field (Sevatdal and Horsberg, 2003; Westcott et al., 2008). Whenever possible, standard bioassays are performed with preadult-II female and preadult-II male salmon lice. These stages are selected because they appear at the same time in synchronised cohorts and have approximately the same size (Sevatdal et al., 2005a). Moreover, preadult-II females can be expected to be physiologically more homogenous than adult females, which undergo post-moulting growth and cycles of egg production and vitellogenesis (Eichner et al., 2008). In bioassays, only healthy individuals must be used. Prior to toxicant exposure, lice must be able to stay attached to the surface of the bioassay container or swim in a straight line. A standard bioassay protocol includes a seawater control and at least five toxicant concentrations, in duplicates (SEARCH Consortium, 2006). Exposure period and recovery time in seawater depend on the toxicant (Table 1.1). Following drug exposure, the behavioural responses of the test individuals are examined and rated. Evaluation criteria differ between working groups, as described in Table S1.1. Usually lice are rated as “live”, “moribund”, and “dead”. If the mortality of lice in the seawater control exceeds 10% bioassay data are usually considered invalid.

Table 1.1 Recommended exposure period and recovery time for different salmon delousing treatments. Data obtained from the SEARCH Consortium (2006).

Active agent	Exposure period	Recovery time
Deltamethrin	30 min	24 h
Azamethiphos	60 min	24 h
Emamectin benzoate	24 h	None

The methodology of bioassays varies between research groups (Table S1.2). For example, salmon lice have been exposed to toxicants in perforated polystyrene boxes placed in aerated seawater (Helgesen and Horsberg, 2013a; Helgesen et al., 2015; Sevatdal et al., 2005a; Sevatdal and Horsberg, 2003), glass flasks (Helgesen and Horsberg, 2013a), Petri dishes (Carmona-Antoñanzas et al., 2016; Igboeli et al., 2012; Westcott et al., 2008), crystallisation dishes (Carmona-Antoñanzas et al., 2017), or directly on Atlantic salmon (Sevatdal and Horsberg, 2003).

Helgesen and Horsberg (2013) and Helgesen et al. (2015) developed single-dose protocols for 24 h bioassays with azamethiphos, emamectin benzoate, hydrogen peroxide, and deltamethrin, which minimise the amount of louse handling and require fewer lice and less effort than

conventional bioassays. The sensitivity of salmon lice can also be monitored in time-to-response bioassays (Carmona-Antoñanzas et al., 2016) in which salmon lice are exposed to the toxicant for 24 h and rated hourly from 1-15 h and at 18, 21, and 24 h after toxicant addition. However, time-to-response bioassays are very time-consuming and therefore not feasible on an industrial scale. Instead of preadult-II/adult salmon lice, bioassays can also be performed with copepodid larvae (Andrews and Horsberg, 2020; Poley et al., 2016).

Although pesticide resistance is traditionally assessed in bioassays, this methodology has several limitations. Standard bioassays with preadult-II/adult require a large number of parasites to meet the required number of concentrations and replicates. They are labour intense and time consuming because several dilutions have to be prepared (Marín et al., 2015). Moreover, the test sensitivity is comparatively low, as the outcome of the experiment is only measured by the behavioural response of the parasite ("dead" or "alive"), which requires expertise (Aaen et al., 2015). In addition, sea lice rating criteria are still inconsistent (Table S1.1). Bioassays are also biased by the executing person, equipment, and experimental set-up (Aaen et al., 2015). Although attempts were made to standardise bioassay protocols variations in methodology exist between research groups (Table S1.2), complicating comparisons of results. Another difficulty is that the adsorption of pesticides varies with different surface materials, which are not yet standardised (Helgesen and Horsberg, 2013b). For example, deltamethrin concentration after 24 h exposure was highest in glass bottles but below the detection limit in polypropylene containers. Moreover, bioassay results may vary depending on whether they are performed with the commercial product (e.g., AMX®) or its active pharmaceutical ingredient (e.g., deltamethrin). Diluting the commercial product is closer to field conditions but less accurate as the proportions of active ingredient and solvent are unknown to the researcher. In addition, bioassays are prone to failure due to interacting environmental factors (Robertson et al., 2017). For example, sea lice that died due to unfavourable temperatures or salinities are easily mistaken for being susceptible to the toxicant. Nonetheless, despite their limitations, bioassays still play a crucial role in phenotyping resistance.

1.5.2 Molecular methods

The need for more accurate, faster, and cheaper methods for the detection of pesticide resistance promoted research on molecular tests. Aaen et al. (2015) reviewed genomic, transcriptomic, and proteomic approaches, which can be used to identify suitable molecular markers for resistance.

To date, PCR-based genotyping tests are marketed to detect resistance towards pyrethroids, azamethiphos, and hydrogen peroxide. With regards to pyrethroids resistance, the test classifies *L. salmonis* as resistant if at least one of the five SNPs T8605G, A9035G, C13957T, A14017G, C14065T (NCBI accession number AY625897.1) is present in the mitochondrial genome (mtDNA) sequence (Nilsen and Espedal, 2015).

1.6 Drug resistance

1.6.1 Evolution of resistance

Drug resistance can be defined as "[…] genetically based decrease in susceptibility to a pesticide" (Tabashnik et al., 2014). Resistance development to chemical treatments is an evolutionary process by natural selection. Genetic variants conferring fitness advantages under pesticide exposure arise by mutations and remain at low frequencies in the absence of pesticides (Ffrench-Constant, 2007). A pesticide is a selection pressure; individuals possessing genes conferring fitness advantages under its exposure are more likely to survive and reproduce. Selection of resistance is a process that requires many generations. If resistance is unchallenged, resistance alleles can reach a high proportion in a population. In the worst case, all individuals within a population become homozygous for a resistant gene and thus, this gene becomes fixed within the population (Kunz and Kemp, 1994; SEARCH Consortium, 2006).

From a theoretical point of view, evolution of resistance is conceivable if (I) treatments are not 100% efficient, (II) genetic determinants exist that increase the fitness of the individual in the presence of treatments, and (III) treatments are implemented in a way providing sufficient selection pressure such as insufficient rotation with other control methods (reviewed by Coates et al., 2021b).

Resistance alleles have either a single origin or several independent origins (Ffrench-Constant et al., 2004). For example, it has been suggested that azamethiphos resistance in *L. salmonis* evolved from multiple independent origins that probably existed in salmon lice before the introduction of azamethiphos in commercial aquaculture (Kaur et al., 2017). In contrast, the analysis of conserved haplotypes across samples from the Atlantic strongly suggests that emamectin benzoate resistance developed at a single source and rapidly spread across the Atlantic Ocean (Besnier et al., 2014).

1.6.2 Factors promoting resistance

Rapid evolution of pesticide resistance is favoured by a short generation time and a large population size, a lack of refuges for susceptible individuals, low fitness costs associated with resistance, and strong selection pressures, e.g., frequent treatments with a limited range of compounds (reviewed by Coates et al., 2021b).

Generation time and population size: Evolution of pesticide resistance is favoured by a short generation time as species with a short generation time tend to have a greater rate of mutations per year than species with a long generation time. In addition, a large population size increases the likelihood for the presence of rare resistance-conferring mutations (Thomas et al., 2010).
L. salmonis has a short generation time ranging from 6 weeks at 9-12°C to 7.5 weeks at 10°C (Johnson and Jakob, 2012), as well as a high reproductive output (Heuch et al., 2000). Moreover, population genetic studies provide evidence for a single panmictic population of *L. salmonis* throughout the entire North Atlantic Ocean (see section 1.2.1) (Besnier et al., 2014; Glover et al., 2011), which favours to the rapid dispersal of initially rare resistance-conferring mutations. Further contributing to the large population size of *L. salmonis* in the North Atlantic is the high number of farmed hosts, which are available year-round and greatly outnumber wild salmonids (Fjørtoft et al., 2021, 2020).

Refuges for susceptible individuals: Selection of resistance is further promoted by a lack of refuges for susceptible individuals (Kreitzman et al., 2018; McEwan et al., 2015; Murray, 2011). In contrast to farmed salmon, wild salmonid hosts are not subject to sea lice management measures and thus, provide a refuge where there is no selection for resistance and non-resistance alleles can be preserved. Theoretically, these refuges can slow down resistance development. A study using agent-based modelling found that resistance formation in salmon lice can be prevented when wild salmonid populations are equal of greater in number than farmed fish (McEwan et al., 2015). While farmed salmon outnumber wild salmonids in the North Atlantic, wild hosts far outweigh farmed salmonids in the North Pacific. Accordingly, Kreitzman et al. (2018) modelled conditions in the Pacific and found that wild salmonids play a key role in delaying resistance development.

Fitness costs associated with resistance: Evolution of resistance is further accelerated when associated with no/low fitness costs (Coates et al., 2021b). A study using agent-based modelling found that even small fitness costs of resistance significantly slow down resistance formation in salmon lice, particularly when farmed hosts outnumber wild hosts, as is the case in the North Atlantic (McEwan et al., 2015).

Fitness costs refer to a trade-off in which alleles conferring increased fitness under the presence of a toxicant reduce fitness in the absence of that toxicant (Bass, 2017; Coustau et al., 2000). Measured fitness components include growth, development time, fertility, fecundity, mating competitiveness, and defence against parasitoids (Freeman et al., 2021; Kliot and Ghanim, 2012). Evidence for fitness costs is provided by studies carried out in a variety of arthropod orders (reviewed by Kliot and Ghanim, 2012). However, other studies failed to detect such costs (Bielza et al., 2014; Castaneda et al., 2011; Elard et al., 1998).

Coustau et al. (2000) suggested that fitness costs depend on the mechanism that is conferring resistance. Fitness costs are expected when resistance is conferred by energetically expensive constitutive overproduction of detoxification enzymes, while no fitness costs seem to be associated with resistance by inducible regulation of gene expression (Field, 2000; Field et al., 1999).

Fitness costs can be compensated by epistasis, meaning that the phenotypic effect of the resistance gene is masked by further mutations at other loci that act as 'modifiers' mitigating fitness costs (Coustau et al., 2000). For example, in the Australian sheep blowfly *Lucilia cuprina* resistance towards the organophosphate diazinon is conferred by a missense mutation in esterase 3 (Hartley et al., 2006), which enables it to hydrolyse organophosphates but abolishes its carboxylesterase (CaE) activity. The resistance has fitness costs that are compensated by a single modifier gene on a different chromosome (Batterham et al., 1996). In addition, genetic changes over several generations can alleviate deleterious effects of resistance mutations (Kliot and Ghanim, 2012). For example, in *C. pipiens* a missense mutation in *ace1* encoding (acetylcholinesterase) AChE is conferring resistance to organophosphates and carbamates but is reducing the AChE enzyme activity by more than 60% when present in homozygous form. Independent duplications of *ace1* consisting of one susceptible and one resistant copy result in a fixed heterozygous phenotype that displays the same resistance level, but reduced fitness costs compared to the homozygous phenotype (Labbé et al., 2007). In some cases, no fitness costs were found when resistance-associated polymorphisms pre-existed in high frequencies before pesticide introduction. For example, in *L. cuprina* organophosphate resistance is conferred by the mutation W251L in esterase 3. The pre-adaptive occurrence of that mutation prior to the introduction of organophosphate enabled the enzyme to retain much of its CaE activity and thus, no fitness costs have been documented (Hartley et al., 2006).

Selection pressures: Over-reliance on a limited range of treatments also facilitates selection for resistance (McEwan et al., 2016). When a treatment is reapplied despite part of the population being resistant further treatments continuously select for resistance alleles and steadily remove susceptible individuals from the population (SEARCH Consortium, 2006). Accordingly, during the

last two decades, drug resistance in sea lice has been promoted by the usage of increasing quantities of a small number of medicinal compounds with declining efficacies (SEARCH Consortium, 2006). Selection for resistance is further promoted by frequent parasite treatments, which can result from statutory treatment thresholds and regular harvests (Mennerat et al., 2017, 2012, 2010). These factors exert specific selection pressures that drive the evolution of faster parasite life history in the farmed environment. Moreover, drugs with long half-lives and/or incorrectly applied treatments can favour the selection of highly resistant animals (SEARCH Consortium, 2006).

1.6.3 Molecular mechanisms of pesticide resistance

Resistance of arthropods to pesticides has been shown to involve two main mechanisms, target-site insensitivity and enhanced detoxification (Hemingway and Ranson, 2000). In addition, reduced cuticular penetration (Koganemaru et al., 2013) and changes in behaviour can contribute to resistance (Silverman and Bieman, 1993).

Target-site insensitivity: Target-site insensitivity is conferred by point mutations in genes coding for proteins constituting pesticide target-sites, which cause changes in the amino acid sequence that disrupt pesticide binding. Such mutations can occur in (1) ion channels/receptors involved in neurotransmission such as glutamate-gated chloride channels (avermectin resistance) (Dermauw et al., 2012), voltage-gated sodium channels (Na_v) (pyrethroid resistance) (Dong et al., 2014), gamma-Aminobutyric acid (GABA) gated chloride channels (organochlorine resistance) (Hope et al., 2010) or nicotinic acetylcholine receptors (neonicotinoid resistance) (Liu et al., 2005), (2) enzymes involved in neurotransmission such as AChE) organophosphate resistance) (Brown and Bryson, 1992; Kaur et al., 2015b, 2015a; Morton and Holwerda, 1985; Tripathi and O'Brien, 1973), (3) protein complexes of the mitochondrial electron transport chain (bifenazate resistance) (Van Leeuwen et al., 2008; Van Nieuwenhuyse et al., 2009), (4) receptors on the brush boarder membrane of gut cells (resistance to crystalline proteins produced by *Bacillus thuringiensis*) (Ferré and Van Rie, 2002), (5) the enzyme chitin synthase (benzoylurea resistance) (Van Leeuwen et al., 2012), and (6) lipid biosynthetic enzymes (spiromesifen resistance) (Karatolos et al., 2012).

Enhanced detoxification: Pesticide resistance can also result from detoxification by enzymes that break-down or sequester the pesticide before it reaches its target-site or transporters that enhance the excretion of the pesticide from the cell. Four groups of detoxifying proteins have been shown to be involved in resistance in insects, namely cytochrome P450 monooxygenases (CYPs), serine hydrolases, glutathione-S-transferases (GSTs), and adenosine triphosphate (ATP) binding cassette (ABC) transporters (Ranson et al., 2002).

Reduced cuticular penetration: In arthropods, pesticides are mainly taken up through the cuticle and transported via the haemolymph to the target organs (Sevatdal et al., 2005b). Accordingly, pesticide resistance can involve reduced cuticular penetration, which can result from cuticle thickening or alterations of the cuticle composition (reviewed by Balabanidou et al., 2018) (Vinson and Law, 1971) (Patil and Guthrie, 1979). However, reduced cuticular penetration is believed to be only a modifier of target-site resistance or increased detoxification rather than a major resistance mechanism (Plapp Jr, 1986; SEARCH Consortium, 2006).

Changes in behaviour: Changes in behaviour have been shown to reduce the efficacy of formulated toxicants in *Blattella germanica* (Hostetler and Brenner, 1994; Wang et al., 2004). In this species, avoidance of ingesting toxicant/diet mixtures has been attributed to an aversion to D-glucose in the diet, which is inherited as an autosomal incompletely dominant trait (Silverman and Bieman, 1993).

Pesticide resistance is often the result of multiple resistance mechanisms (Ffrench-Constant, 2013; Heckel, 2012). For example, in *Musca domestica* 10-fold increased GST activity, 5-fold increased CaE activity, and 10-fold reduced sensitivity of AChE against organophosphate inhibition led to 519-fold increased resistance to malathion (Yeoh et al., 1981). The number of resistance genes under pesticide selection depends on whether selection acts within or outside of the normal response of a population (Ffrench-Constant et al., 2004). Selection within a continuous phenotypic distribution, e.g., small laboratory population, favours selection of polygenic resistance based on different genes, most of which have small effects on susceptibility (Ffrench-Constant, 2013; McKenzie and Batterham, 1994). In contrast, selection outside the phenotypic distribution favours monogenic resistance based on rare mutations having a large effect on susceptibility. For example, insecticides used for crop protection are typically applied at levels well above those tolerated by pests as adverse side effects on crops are not expected. Thus, only individuals carrying rare mutations that decrease susceptibility by a large extent will be able to survive, resulting in monogenic resistance (Ffrench-Constant, 2013; McKenzie and Batterham, 1994). In contrast, many anti-parasitic drugs show only moderate differential toxicity between the parasite and the protected species (Roth, 2000), necessitating dose limitations to avoid detrimental effects on non-target species (Aaen et al., 2015). As a result, relatively low levels of resistance in the parasite can become clinically relevant, potentially facilitating the evolution of polygenic resistance.

Multiple resistance differs from cross-resistance, which refers to "[…] a situation in which a strain that becomes resistant to one insecticide automatically develops resistance to other insecticides to which it has not been exposed" (Simon, 2014). Cross-resistance can result from

structural alterations of the pesticide's target-site that reduce the sensitivity towards other drugs that bind to the same site, e.g., target-site mutations in Na_v (Carnevale and Guillet, 1999; Chandre et al., 1999), enhanced detoxification by non-specific enzymes that target functional groups of insecticides, e.g., CYPs (Daborn et al., 2001; Yunta et al., 2019), or reduced cuticle penetration, which can affect chemically unrelated compounds (Simon, 2014).

1.7 Pyrethroids

1.7.1 Chemical properties

Based on their chemical structure, pyrethroids can be divided into two groups, type I and type II pyrethroids. Type II pyrethroids contain an alpha-cyano-3-phenoxybenzyl group and include deltamethrin, cypermethrin and fenvalerate. Type I pyrethroids lack the alpha-cyano-3-phenoxybenzyl group and comprise a wider structural variety. For example, they include permethrin, allethrin, tetramethrin, and resmethrin (Fig. 1.7).

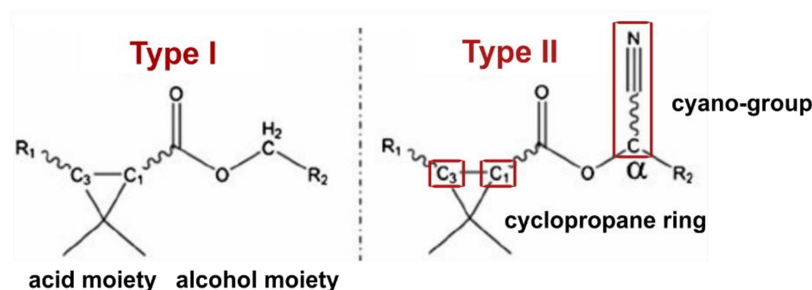

Figure 1.7 General structure of type I and type II pyrethroids according to Corcellas et al. (2015).
The C_1, C_3, and α-cyano-group are highlighted.

Pyrethroids exist in two geometric isomers, depending on whether the two substituents of the cyclopropane ring at C_1 and C_3 are oriented in the same direction (Z/cis) or on opposite sides (E/trans) (Simon, 2014). In addition, pyrethroids also occur as enantiomers because they have two chiral centres at C_1 and C_3, which can exist as either R or S form (1R or 1S; 3R or 3S). Type II pyrethroids have a third chiral centre at the α-carbon that can also be designates as αR or αS.

The substituents and stereochemical characteristics of the acid and alcohol moiety affect the insecticidal activity of pyrethroids (Casida, 1980). While both Z/cis and E/trans isomers are biological active, only the 1R and αS forms are biological active and toxic (Elliott et al., 1978; Simon, 2014). Type II pyrethroids are usually more toxic than type I pyrethroids because the cyano-group retards their metabolism (Casida, 1980). The insecticidal toxicity is further enhanced by replacement of the isobutenyl group with a dihalovinyl group, such as a dibromvinyl group in deltamethrin (Ruzo and Casida, 1977).

1.7.2 Use of pyrethroids

Pyrethroids are widely used to control insects that are phytophagous, parasitic, or represent vectors for human disease (Zhang, 2018). They are active ingredients in chemicals for outdoor use in agriculture and aquaculture, as well as in insecticides for use in and around the home (Katsuda, 2011). The first pyrethroid, allethrin, was developed in 1949 and in the 1970s production of pyrethroids for agricultural use started in Europe, the USA, and Japan (Katsuda, 2011). In aquaculture, pyrethroids are only used since the mid-1990s (Aaen et al., 2015).

1.7.3 Deltamethrin: Chemical and physical properties

Deltamethrin is a type II pyrethroid. Its IUPAC name is [(S)-cyano-(3-phenoxyphenyl)methyl] (1R,3R)-3-(2,2-dibromoethenyl)-2,2-dimethylcyclo-propane-1-carboxylate ($C_{22}H_{19}Br_2NO_3$) (Fig. 1.8).

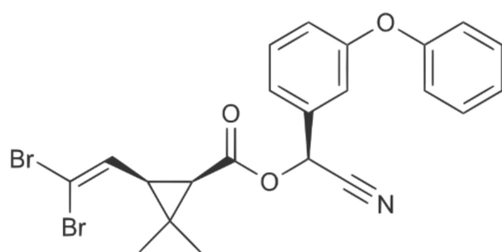

Figure 1.8 Structure of the pyrethroid deltamethrin.

Deltamethrin is very hydrophobic (water solubility: 0.002 mg L^{-1} at 25°C). The compound has a high octanol/water partition coefficient ($\log K_{ow} = 6.2$) (Hansch et al., 1995) and thus, binds strongly and quickly to organic material within the water column, such as sediment or the body surface of sea lice and fish in bath treatments. The half-life of deltamethrin in water is 2-4 h (Muir et al., 1985). It is highly degradable in unbound form in the environment (Muir et al., 1985). However, when bound, it is very stable and degrades slowly under both aerobic and anaerobic conditions.

1.7.4 Commercial product AMX®

The veterinary drug AMX® (PHARMAQ AS) contains 10 mg mL^{-1} of the active ingredient deltamethrin. AMX® is used as a bath treatment to remove preadult and adult *L. salmonis* from Atlantic salmon and rainbow trout. It is recommended to administer 0.2 mL AMX® per m^3 seawater (2 µg L^{-1}) for a period of 30 min. The withdrawal period for treated Atlantic salmon and rainbow trout is five degree-days (PHARMAQ AS, 2017).

AMX® is approved in the United Kingdom since 2008 and in Ireland since 2006. In Norway and the Faroe Islands AMX® is distributed under the name ALPHA MAX® (PHARMAQ AS) since 1998 and

1997, respectively. In Chile, AMX® Deltametrina 10 mg mL^{-1} (PHARMAQ AS) is approved since 2007.

To avoid hypoxemia in treated fish (Grant and Davis, 2000), oxygenation has to be provided to keep the oxygen concentration above 7 mg L^{-1} during the entire procedure (PHARMAQ AS, 2017). Increases of the dose or treatment time beyond the recommendation can lead to side effects in fish (Roth, 2000). In laboratory trials, signs of intoxication of fish have been seen with doses three times the recommended dose at 60 min exposure and five times the recommended dose at 30 min (PHARMAQ AS, 2017). Moreover, the toxicity of pyrethroids also depends on the water temperature (Kumaraguru and Beamish, 1981). At water temperatures below 6˚C both the toxicity and the safety margin of AMX® decline (PHARMAQ AS, 2017).

1.8 Mode of action of deltamethrin

1.8.1 Effects on voltage-gated sodium channels

Voltage-gated sodium channel are large membrane proteins that are responsible for the initiation of action potentials in nerve, muscle, and other excitable cells by mediating an increase in sodium ion permeability (Catterall, 2000).

Mammalian Na_v consist of the large pore-forming α-subunit Na_v1 and auxiliary β-subunits (Catterall, 2000). Na_v1 is functional on its own. It comprises four internally homologous domains DI-IV (Fig. 1.9A), each consisting of six membrane spanning segments (S1-S6). The S4 segments contain positively charged amino acids, which serve as a voltage sensor. S5, S6, and an extracellular loop connecting S5 and S6, called the P-region, form the pore module (Fig. 1.9B). The cytoplasmic linker (L) between DIII and IV contains a hydrophobic sequence motif (isoleucine (Ile, I); phenylalanine (Phe, F); methionine (Met, M); IFM motif), which serves as an inactivation-gate that can block the inner mouth of the pore (Rohl et al., 1999). Opening and closing of the inactivation-gate and the voltage-dependent activation-gate initiate conformational changes of Na_v1 (closed state, open state, inactivated state, deactivated state), which mediate the action potential (Barnett and Larkman, 2007; Davies et al., 2007a).

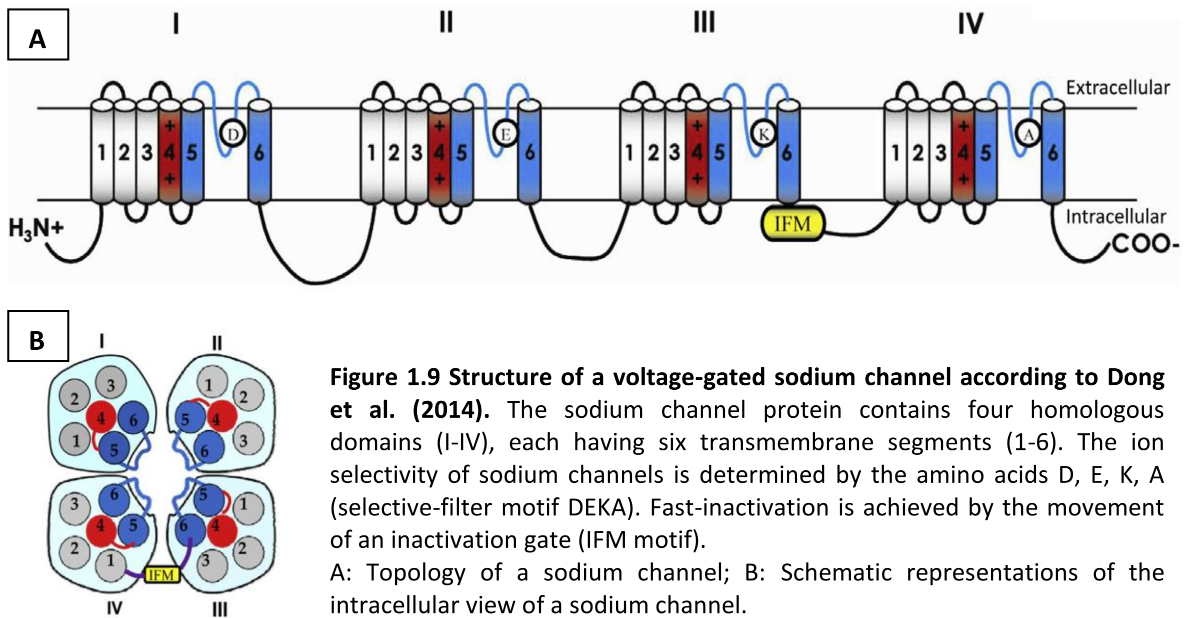

Figure 1.9 Structure of a voltage-gated sodium channel according to Dong et al. (2014). The sodium channel protein contains four homologous domains (I-IV), each having six transmembrane segments (1-6). The ion selectivity of sodium channels is determined by the amino acids D, E, K, A (selective-filter motif DEKA). Fast-inactivation is achieved by the movement of an inactivation gate (IFM motif).
A: Topology of a sodium channel; B: Schematic representations of the intracellular view of a sodium channel.

Insect voltage-gated sodium channels share high levels of structure, amino acid sequence, and function with the mammalian α-subunit Na_v1. The first insect Na_v1 gene was cloned from *Drosophila melanogaster* (*para* gene, later named $DmNa_v$) (Loughney et al., 1989). Its channel function was subsequently confirmed by expression and characterisation in *Xenopus* oocytes (Feng et al., 1995; Warmke et al., 1997). While mammals achieve functional diversity of Na_v1 by expression of α-isoforms with different gating properties (Goldin et al., 2000), most insects only have a single Na_v1 gene whose activity is diversified through alternative splicing (Loughney et al., 1989; Thackeray and Ganetzky, 1995, 1994) and RNA editing (Hanrahan et al., 2000; Olson et al., 2008; Reenan et al., 2000).

Historically, pyrethroids have been classified based on their symptomology in rats into compounds causing whole body tremor (T-syndrome) or choreoathetosis with salivation (CS-syndrome) (Verschoyle and Aldridge, 1980). Although this classification is not absolute, type I pyrethroids are usually characterised by the T-syndrome and type II pyrethroids by the CS-syndrome (Wright et al., 1988). Subsequent studies focused on defining the neurotoxic action of pyrethroids to explain the different poisoning syndromes (Soderlund and Bloomquist, 1989). Electrophysiological experiments in frog, squid, and crayfish revealed that type I pyrethroids cause repetitive firing in axons followed by a block in nerve conduction in response to a single stimulus (Lund and Narahashi, 1983; Murayama et al., 1972; Narahashi and Anderson, 1967; Vijverberg and Berecken, 1982). They induce a slight prolongation of the open time of the channel (i.e., sodium tail currents of ~20 ms), leading to multiple long action potentials (Schleier III and Peterson, 2011). In contrast, electrophysiological experiments with type II pyrethroids revealed stimulus-dependent membrane depolarisation and conduction block (Vijverberg and

Berecken, 1982). They significantly prolong the channel open time (i.e., sodium tail currents of ~200 ms), resulting in increased resting membrane potential and often depolarisation-dependent block of action potentials (Schleier III and Peterson, 2011). While type II pyrethroids primarily bind to the open state of Na_v1, type I pyrethroids have been shown to modify closed Na_v1 (Soderlund and Knipple, 2003). However, both types of pyrethroids impede closing of Na_v1 by inactivation or deactivation, resulting in an influx of Na^+ into the neuron. As the membrane potential shifts, the neuron functions in a relatively stable state of abnormal hyperexcitability. In arthropods, this causes a sublethal effect called 'knockdown'. The inward current of Na^+ continues until the level of hyperexcitability overwhelms the capacity of the cell to maintain the activity of the Na^+-K^+ pump (Davies et al., 2007a). As type II pyrethroids delay the inactivation of Na_v1 substantially longer than type I pyrethroids, they are more potent toxicants.

Homology modelling of insect sodium channels and ligand docking studies revealed two pyrethroid binding sites, docking onto which stabilises the channel in the open state. Site 1 involves L4-5 and S5 of DII and S6 of DIII (O'Reilly et al., 2006), while Site 2 maps to L4-5 and S5 of DI and S6 of DII (Du et al., 2013). DTT and several type I and type II pyrethroids have been docked into Site 1 (O'Reilly et al., 2006; Usherwood et al., 2007), and most pyrethroid-sensing residues in Site 1 were found to have analogues in Site 2 (Du et al., 2013). This indicates that both sites are rather symmetric, although not identical (Du et al., 2013). Docking studies also revealed different contact points of type I and type II pyrethroids with the two bindings sites (Usherwood et al., 2007). For example, the α-cyano-group of specific type II pyrethroids provides an additional contact point with the channel, which restricts its conformation to a more optimal pose for binding (O'Reilly et al., 2006; Usherwood et al., 2007). Docking studies also provide insights into structural and stereospecific characteristics for pesticidal action (O'Reilly et al., 2006). While the acid moiety of a pyrethroid tolerates large degrees of substitutions before insecticidal activity is lost, the stereochemical characteristics of the central region around the ester linkage and the orientation of the alcohol moiety are more critical for the action of pyrethroids.

1.8.2 Additional molecular target sites of pyrethroids

The primary target-site of the pyrethroid deltamethrin is widely accepted as Na_v1 (Dong et al., 2014). However, due to their lipophilic nature pyrethroids can pass and interact with biological membranes, making a variety of other membrane proteins and structures candidate targets for pyrethroid action (Guven et al., 2018).

In addition to their effect on Na_v1, type II pyrethroids can modify the gating kinetics of voltage-gated calcium channels (VGCC) (Clark and Symington, 2008). VGCC mediate Ca^{2+} influx into the cell following membrane depolarisation, which can alter cell signalling, neurotransmission, and gene expression (reviewed by Catterall, 2011). Deltamethrin was found to reduce the peak current of a rat VGCC isoform expressed in *Xenopus* oocytes in a concentration-dependent and stereospecific manner and caused a prolongation of the channel's activation and inactivation kinetics (Clark and Symington, 2008, 2007; Symington and Clark, 2005). In addition, deltamethrin increased Ca^{2+} influx and Ca^{2+}-dependent neurotransmitter release. Pyrethroids were found to modify mammalian neuronal VGCCs at concentrations similar to Na_v1 (Hildebrand et al., 2004; Neal et al., 2010).

Type II pyrethroids have also been shown to bind to GABA-type A ($GABA_A$) receptor-gated chloride channels, which inhibits GABA dependent uptake of Cl^-, leading to hyperexcitability and neurotoxicity (Bloomquist and Soderlund, 1985; Kumar Singh et al., 2012; Lawrence and Casida, 1983). In electrophysiological experiments using crayfish claw opener muscles, type II pyrethroids increased the input resistance of the claw opener muscle in a manner similar to the $GABA_A$ receptor antagonists picrotoxin (Gammon and Casida, 1983). However, further studies suggested that effects of pyrethroids on $GABA_A$ receptors are indirect and downstream of the effects on Na_v1. While deltamethrin, cypermethrin and permethrin induced a concentration-dependent decrease in GABA-dependent $^{36}Cl^-$ influx this effect was completely inhibited by tetrodotoxin, a toxin that blocks the action of pyrethroids on Na_v1 (Eshleman and Murray, 1991).

Another secondary site of action for a subset of pyrethroids are voltage-gated chloride channels (CLC) (reviewed by Wakeling et al., 2012), which perform important roles in the regulation of cell volume, acidification of intracellular organelles, stabilisation of resting membrane potentials and transepithelial membrane transport (Jentsch et al., 1999). For example, deltamethrin and bioallethrin decreased the probability of CLC opening in cultured mouse neuroblastoma cells, whereas esfenvalerate and cyhalothrin exposure induced no effect (Burr and Ray, 2004; Forshaw et al., 1993; Ray et al., 1997). The CLC agonist ivermectin increased the probability of CLC opening in deltamethrin-treated cultured mouse neuroblastoma cells and caused a marked fall in deltamethrin-induced salivation and repetitive muscle twitching (Forshaw et al., 2000).

Type II pyrethroids have also been shown to affect nicotinic acetylcholine receptors (Abbassy et al., 1983a, 1983b, 1982; Oortgiesen et al., 1989) and glutamate receptors (Frey and Narahashi, 1990; Staatz et al., 1982). Moreover, deltamethrin was also found to decrease the AChE activity in brain of rats (Khan et al., 2018; Saoudi et al., 2017), which has been attributed to a reduction

of the acetylcholine binding space due to interaction of the drug with the aromatic, hydrophobic enzyme surface (Khan et al., 2018, 2013).

Due to their lipophilic nature, pyrethroids can also affect mitochondrial membrane structures and dynamics (Braguini et al., 2004; Zhang et al., 2007) and can induce inhibition of mitochondrial respiratory complexes (Braguini et al., 2004; Gassner et al., 1997), which can disrupt the mitochondrial oxidative phosphorylation. In isolated rat liver mitochondria, permethrin and cyhalothrin caused inhibition of respiratory complex I (Gassner et al., 1997), and deltamethrin was predicted to have a major inhibition site between respiratory complexes II and III (Braguini et al., 2004). Mitochondrial oxidative phosphorylation can also be impaired by intracellular Ca^{2+} accumulation (Bauer and Murphy, 2020; Paschen, 2000), which can result from interactions of pyrethroids with Na_v1 and consequent Ca^{2+} influx (Hossain and Richardson, 2011) and direct effects of pyrethroids on VGCC (reviewed by Clark and Symington, 2011). Effects are further enhanced by energy deficits to remove cytosolic Ca^{2+}, which can result from an increasing demand for ATP when ATP-driven sodium channels are inhibited (Chinopoulos et al., 2000) or direct inhibition of mitochondrial respiratory complexes by pyrethroids.

Type I and type II pyrethroids have also been shown to induce the generation of reactive oxygen species (ROS; collectively superoxide radicals O_2^-, hydrogen peroxide H_2O_2, hydroxyl radicals OH^-) in both the cytosol and the mitochondria (Klimek, 1990; Truong et al., 2006; Vontas et al., 2001). Overproduction of ROS can trigger a cascade of reactions that induces lipid peroxidation and damage of macromolecules (Chirico et al., 1993; Fang et al., 2002; Maiti et al., 1995). Furthermore, ROS-mediated damage of macromolecules within the mitochondria can lead to disruption of oxidative phosphorylation (Fariss et al., 2005). In the cytosol, ROS can be generated during CYP-mediated oxidative metabolism of pyrethroids (Klimek, 1990; Vontas et al., 2001), and within the mitochondria pyrethroids can trigger the formation of ROS through disruption of the mitochondrial membrane integrity and inhibition of respiratory complexes (Sipos et al., 2003; Truong et al., 2006). In addition, ROS can be generated via pyrethroids induced intracellular Ca^{2+} accumulation (Brookes et al., 2004).

Pyrethroids have also been shown to induce apoptosis in different mammalian cell types by mechanisms involving intrinsic (mitochondrial) (Ko et al., 2016; Kumar et al., 2016) and extrinsic (death receptor) pathways (Arslan et al., 2017), as well as the ER stress pathway (Hossain and Richardson, 2011). The intrinsic mitochondrial pathway involves mitochondrial outer membrane permeabilization (MOMP), release of cytochrome C (cytC) into the cytosol, binding of cytC to apoptotic peptidase activating factor 1, activation of caspases, and ultimately DNA fragmentation (Bock and Tait, 2020). MOMP is considered a point-of-no-return in apoptosis

execution (Kroemer, 2003). Among others, it can be triggered by pyrethroid induced oxidative stress and Ca^{2+} accumulation (Buttke and Sandstrom, 1994; Hajnóczky et al., 2003; Lenaz, 1998). The mitochondrial apoptotic pathway can also be induced by low levels of ATP that lead to disruption of the mitochondrial transmembrane potential. In addition, increased mitochondrial permeability and apoptosis has been linked to enhanced expression of pro-apoptotic p53 and B-cell lymphoma 2-associated X protein (Bax), and decreased expression of anti-apoptotic B-cell lymphoma 2 (Bcl-2) following deltamethrin exposure (Wu et al., 2000; Wu and Liu, 2000a, 2000b).

1.9 Mechanism of pyrethroid resistance in terrestrial arthropods

1.9.1 Target site mutations in voltage gated sodium channels

Knockdown resistance (*kdr*) is a common mechanism of pyrethroid resistance in arthropods, which results from non-synonymous point mutations in Na_v1. These mutations decrease the potency of pyrethroid by altering the Na_v1 gating kinetics and/or by impairing pyrethroid binding to the channel. The decreased affinity of Na_v1 for pyrethroids increases the dissociation rate of the pyrethroid-Na_v1-complex and enhances the entry of Na_v1 into the inactive state (Soderlund and Knipple, 2003).

The inheritance of the *kdr* resistance phenotype is not yet fully understood, but most studies suggest incompletely recessive inheritance of the resistance trait (Foil et al., 2005; Halliday and Georghiou, 1985; Hopkins and Pietrantonio, 2010; Stenhouse et al., 2013), meaning that heterozygous individuals are slightly more resistant than the homozygous wild type and less resistant than the homozygous mutant (Stone, 1968).

The first *kdr* trait was documented in *M. domestica* by Busvine et al. (1951) and eventually mapped to substitution L1014F (Numbered according to NCBI accession no. AAB47604) (Ingles et al., 1996; Miyazaki et al., 1996; Williamson et al., 1996, 1993). A second resistance trait in *M. domestica*, M918T, was found to confer much greater resistance than *kdr* and thus, designated *super-kdr* (Sawicki, 1978; Williamson et al., 1996). To date, more than 50 *kdr*-type mutations have been described and many of them have been functionally confirmed to be associated with pyrethroid resistance by recombinant expression in *Xenopus* oocytes (reviewed in Dong et al., 2014). Most *kdr*-type mutations were found to cluster within the predicted pyrethroid-binding sites: Site 1 involving L4-5 and S5 of DII and S6 of DIII (O'Reilly et al., 2006), and Site 2 comprising L4-5 and S5 of DI and S6 of DII (Du et al., 2013). For example, M918T (DII S4-5), L925I (DII S5), and T929I (DII S5; numbered according to NCBI accession no. AAB47604) within Site 1 reduced pyrethroid sensitivity when expressed in *Xenopus* oocytes and docking studies with fenvalerate revealed close binding contacts (<4 Å) with these residues

(O'Reilly et al., 2006). *Kdr* mutation L1041F was predicted to lie within Site 2 (DII S6), in an analogous position to a pyrethroid-sensing residue within Site 1, and also reduced pyrethroid sensitivity (Du et al., 2013).

Kdr mutations often confer cross-resistance between two different pyrethroids and DTT, indicating shared Na_v1 binding sites between these compounds (Schleier et al., 2012; Usherwood et al., 2007). For example, mutation T929I in *D. melanogaster* increased resistance to deltamethrin, permethrin, fenfluthrin, and DDT (Usherwood et al., 2007).

Several *kdr* mutations have evolved independently in different arthropod species, such as mutations L1041F and M981T in *M. domestica*, *Haematobia irritans irritans, Liriomyza huidobrensis, Myzus persicae, Thrips tabaci*, and *Tuta absoluta* (Eleftherianos et al., 2008; Guerrero et al., 1997; Haddi et al., 2012; Toda and Morishita, 2009; Usherwood et al., 2007; Williamson et al., 1996). Moreover, co-occurrence of more than one *kdr*-type mutation has been shown to decrease the Na_v1 sensitivity to pyrethroids compared to individual mutations (reviewed by Dong et al., 2014). For example, L1041F and M981T alone induced 5- to 10-fold decrease in deltamethrin susceptibility of $DmNa_v$, whereas the double mutation L1041F + M981T almost abolished the sensitivity of $DmNa_v$ to deltamethrin (Lee et al., 1999; Vais et al., 2000).

1.9.2 Increased detoxification

1.9.2.1 Cytochrome P450 monooxygenases

CYPs are best known for their monooxygenase role. They bind molecular oxygen and receive electrons from NADPH to introduce an oxygen molecule into endogenous and foreign chemical substrates (Feyereisen and Gilbert, 2012). Accordingly, CYPs can catalyse the hydroxylation of deltamethrin at positions 2, 4, or 5 to (2'), 4', or (5')-hydroxy-deltamethrin, which can be further metabolised by certain serine hydrolases (Anand et al., 2006). Metabolism of deltamethrin by hydroxylation results in reduced toxicity as metabolites are either less toxic or cannot reach the site due to decreased stability, changed polarity, or neutralisation (Simon, 2014).

Pyrethroid resistance has been associated with overexpression of CYPs in a variety of arthropod species, such as *Helicoverpa armigera* (Brun-Barale et al., 2010), *Culex quinquefasciatus* (Komagata et al., 2010), and *Helicoverpa zea* (Hopkins and Pietrantonio, 2010).

At the transcriptional level, enhanced CYP activity leading to pyrethroid resistance can be explained by five molecular mechanisms: (I) Upregulation via mutations in cis-acting elements (Itokawa et al., 2010), which modify the transcription of nearby genes (Wittkopp, 2005), (II) upregulation via mutations in trans-regulatory loci (Kasai and Scott, 2001; Liu and Scott, 1996), which modify the transcription of distant genes (Wittkopp, 2005), (III) upregulation via

changes in the coding sequence (Amichot et al., 2004), (IV) upregulation via gene amplification (Wondji et al., 2009), or (V) resistance via a chimeric P450 enzyme that has arisen as the result of gene fusion between two CYP genes by unequal crossing-over, conferring the ability of metabolically inactivating the pyrethroid (Joußen et al., 2012).

1.9.2.2 Serine hydrolases

Esterases: Most serine hydrolases involved in pyrethroid metabolism belong to the CaE gene family (Pfam PF00135 domain), a branch within the α/β-hydrolase fold superfamily (Pfam PF00561 domain) (Punta et al., 2012). The CaE family is functionally diverse. It comprises highly specialised enzymes acting on specific substrates, as well as less-selective enzymes with broad ranges of substrates, and catalytically inactive members with diverse roles including neurodevelopmental signalling or surface recognition (Oakeshott et al., 2005). However, pesticide resistance is believed to be conferred only by catalytically active CaEs, which possess a catalytic triad with a nucleophilic residue (serine (Ser), cysteine (Cys), or aspartic acid (Asp)), an acidic residue (glutamic acid (Glu) or Asp), and a histidine residue (Myers et al., 1988).

Catalytically active CaEs can catalyse the hydrolysis of ester pesticides into their corresponding acid and alcohol metabolites, which are more polar than the parent compound and thus more readily excreted (reviewed by Hemingway, 2000). Accordingly, deltamethrin can be hydrolysed into the less toxic metabolites 3-phenoxybenzoic acid (3-PBA) and deltamethric acid (DA; 3-(2,2-Dibromovinyl)-2,2-dimethyl-cyclopropanecarboxylic acid), and the hydroxylation product (2'), 4', or (5')-hydroxy-deltamethrin can be hydrolysed to DA and 3-(2', 4', or 5'-Hydroxyphenoxy)-benzoic acid (Anand et al., 2006; Day and Maguire, 1990). Catalytically active CaEs have also been shown to mediate resistance by sequestering ester and non-ester pesticides, impairing interactions with their toxicological target-sites (reviewed by Hemingway, 2000).

In terrestrial arthropods, different molecular mechanisms of pesticide resistance involving esterases can be distinguished (reviewed by Hemingway, 2000). Firstly, resistance can be based on the increased expression of esterases following gene amplification (Field and Devonshire, 1998; Rooker et al., 1996). Secondly, constitutive upregulation of CaE gene expression has been implicated in pesticide resistance in several insect species (Zhu and Luttrell, 2015). Finally, single point mutations around the CaEs active site have been shown to confer a change in substrate specificity. Some mutations were found to induce a loss of CaE activity but the acquisition of organophosphate hydrolase activity (Campbell et al., 1998; Claudianos et al., 1999; Newcomb et al., 1997), while others have been shown to enhance the hydrolytic activity of the enzyme for insecticidal isomers of several pyrethroids (Devonshire et al., 2007; Heidari et al., 2005).

Proteases: Besides esterases, certain proteases with a catalytic triad of Ser, histidine (His), and Asp can hydrolyse ester bonds (Perona and Craik, 1995). One of the largest and best characterised serine protease families that meets this criterion is the family S1, which includes trypsin and chymotrypsin (Rawlings and Barrett, 1993). Mass spectrometry data suggested that chymotrypsin splits the ester bond in deltamethrin, although the fragmentation profile of the metabolites was slightly different from cleavage by CaEs (Yang et al., 2008a). Trypsin- and chymotrypsin-like genes were significantly up-regulated in deltamethrin resistant *Culex pipiens pallens* strains, stable transfection of these genes conferred protective effects, and RNAi-mediated knockdown made cells more sensitive to deltamethrin exposure (Gong et al., 2005; Yang et al., 2008b). Serine proteases may also contribute to pesticide resistance by supplying the energy necessary for pesticide mitigation through conformational modification of other detoxifying/target enzymes as part of the induction process (Ahmed et al., 1998). In addition, serine proteases may increase the supply of precursor amino acids for de-novo synthesis of detoxifying enzymes after pesticide treatment (Wilkins et al., 1999).

1.9.2.3 Glutathione-S-transferases and antioxidant enzymes

Another group of metabolic enzymes involved in pesticide resistance are GSTs. They are most commonly involved in the defences against organophosphates (Hemingway et al., 1991) and organochlorines (Lagadic et al., 1993). However, high levels of GSTs have also been linked to pyrethroid resistance in serval arthropod species (Grant and Matsumura, 1989; Reidy et al., 1990) such as *Apis mellifera* (Yu et al., 1984), *B. germanica* (Hemingway et al., 1993), *Spodoptera exigua* (Punzo, 1993), *Anopheles gambiae* (Mueller et al., 2008), and *Tenebrio molitor* (Kostaropoulos et al., 2001).

GSTs are not thought to be involved in the direct metabolism of pyrethroids (Grant and Matsumura, 1989; Reidy et al., 1990). However, they may offer passive protection by binding pyrethroid molecules in a sequestration mechanism (Grant and Matsumura, 1989, 1988; Kostaropoulos et al., 2001). Pyrethroid-binding has been suggested to occur at the active site of the GST enzyme, but not conjugated with glutathione (GSH) (Kostaropoulos et al., 2001). This mechanism decreases the level of free pesticide and may facilitate binding of other pyrethroid-metabolising enzymes with the pesticide. In addition, GSTs with glutathione peroxidase activity can protect tissues against pyrethroid induced oxidative damage (reviewed by Ranson and Hemingway, 2005). For example, a GST has been shown to mitigated pyrethroid induced lipid peroxidation in *Nilaparvata lugens* (Vontas et al., 2001, 2002).

GSTs can confer pyrethroid resistance through up-regulated transcription (Lumjuan et al., 2011) or gene amplification (Vontas et al., 2001, 2002).

Besides GSTs, antioxidant enzymes such as superoxide dismutases (SODs), catalases, and peroxidases can offer protection against pyrethroid-induced oxidative stress. For example, SODs and thioredoxin-dependent peroxidase have been shown to be overexpressed in *Anopheles arabiensis* exposed to deltamethrin (Mueller et al., 2008).

1.9.2.4 ABC transporters

ABC transporter proteins constitute one of the most abundant transporter superfamilies in all organisms from bacteria to humans (Dassa and Bouige, 2001). Prototypical ABC proteins bind ATP and use the energy to translocate various molecules including sugars, amino acids, metal ions, vitamins, sterols, peptides, proteins, hydrophobic compounds, and metabolites across biological membranes (reviewed by Dean et al., 2001). Some of these ABC pumps can reduce the cellular concentration of drugs by directional transport of substrates into excreta and out of sanctuary sites.

ABC transporters have been shown to play an important role in resistance to pyrethroids in several arthropod species such as *Aedes aegypti* (Bariami et al., 2012), *A. sinensis* (He et al., 2019), *A. gambiae* (Bonizzoni et al., 2012), and *A. stephensi* (Epis et al., 2014; Mastrantonio et al., 2017).

1.9.3 Reduced cuticular penetration

Pyrethroid resistance can also be conferred by reduced cuticular penetration, which can result from cuticle thickening or alterations of the cuticle composition (reviewed by Balabanidou et al., 2018). Pyrethroid resistance associated with reduced cuticular penetration has been reported for several arthropod species such as *H. armigera* (Ahmad et al., 2006; Ahmad and McCaffery, 1999; Gunning et al., 1995), *Heliothis virescens* (Ottea et al., 2000), *H. zea* (Abd-Elghafar and Knowles, 1996), *Plutella xylostella* (Noppun et al., 1989), and *M. domestica* (Golenda and Forgash, 1989). For example, a cypermethrin resistant strain of *Bactrocera dorsalis* showed a thicker cuticle, more and denser chitin layers in the endocuticle, more coiled filaments, and thicker epidermal cell interspaces than the susceptible strain (Lin et al., 2012).

1.10 Mechanism of deltamethrin resistance in sea lice

1.10.1 Mutations in the mitochondrial genome

Previous genetic crossing experiments between deltamethrin resistant and susceptible *L. salmonis* have demonstrated a predominately maternal mode of inheritance of deltamethrin resistance (Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015). Carmona-Antoñanzas et al. (2017) crossed deltamethrin resistant female *L. salmonis* with susceptible male *L. salmonis* and vice versa to produce families panning three filial generations. The authors found that deltamethrin resistant females transmitted their resistance to their first filial generation (F1) (EC_{50} >10 µg L^{-1} deltamethrin), F2) (98.8% unaffected by 2 µg L^{-1} deltamethrin, n=173) and F3 progenies (81.2% unaffected by 2 µg L^{-1}, EC_{50} = 9.66 µg L^{-1}) (Fig. 1.10). In contrast, resistant males did usually not transfer their deltamethrin resistance to their progenies (F1: 90% immobilised by 2.5 µg L^{-1} deltamethrin); F2: 83.3% immobilised by 2 µg L^{-1}, n = 84; F3: 81.2% immobilised by 2 µg L^{-1}, EC_{50} = 0.26 µg L^{-1}).

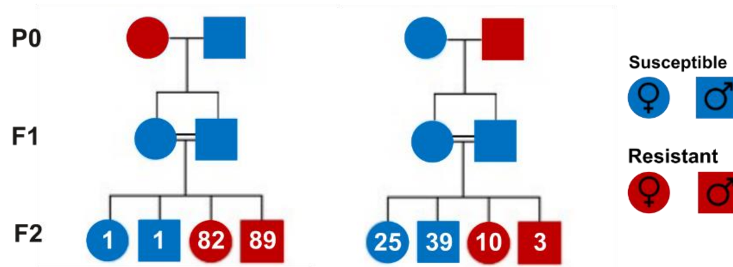

Figure 1.10 Pedigree chart of *L. salmonis* crosses according to Carmona-Antoñanzas et al. (2017). Numbers represent the total number of resistant (red) or susceptible (blue) individuals of each sex. Salmon lice were classified resistance when remaining unaffected after 30 min exposure to 2 µg L^{-1} deltamethrin and 24 h recovery in seawater.

Deltamethrin resistant isolates originating from different regions of Scotland showed virtually identical mtDNA sequences, suggesting the involvement of mitochondrial genes in the resistance mechanisms and clonal expansion of a mitochondrial haplotype associated with increased fitness during pyrethroid treatment (Carmona-Antoñanzas et al., 2017).

The mtDNA from *L. salmonis* comprises 13 gene coding for proteins of mitochondrial complexes I, II, and IV, and the ATP synthase (cytochrome B (CYTB); NADH dehydrogenase (ND) subunits 1-6 and 4L; cytochrome c oxidase (COX) subunits 1-3; ATPase subunits 6 and 8,), as well as 2 rRNAs and 22 tRNAs (Tjensvoll et al., 2005).

MtDNA sequence analyses between deltamethrin resistant and susceptible *L. salmonis* revealed 28 SNPs that were common to all resistant lice but lacking in all susceptible lice (Carmona-Antoñanzas et al., 2017). Four of these SNPs correspond to changes in the protein sequence of ND1 (G8135A [EBI ENA ERP017457] leading to Gly251Ser), ND5 (T5889C leading to Leu411Ser), COX1 (T8600C leading to Leu107Ser), and COX3 (G3338A leading to Gly33Glu), and have been identified by two independent studies (Bakke et al., 2018; Carmona-Antoñanzas et al., 2017). It can be assumed that some of the mitochondrial candidate SNPs described by Carmona-Antoñanzas et al. (2017) and Bakke et al. (2018) are associated with deltamethrin resistance without being involved in its mechanism. Firstly, the mtDNA has a high mutation rate with substitutions being accumulated ten times faster than in nuclear genes (Brown et al., 1979; Tjensvoll et al., 2006), and secondly, the inheritance of mtDNA is linear and lacks recombination through meiosis (Scheffler, 2001).

MtDNA mutations may provide protection against deltamethrin toxicity in *L. salmonis*, which may involve molecular targets encoded by mitochondrial genes (Bakke et al., 2018; Carmona-Antoñanzas et al., 2017). In support of this hypothesis, Bakke et al. (2018) found that the two non-synonymous mutations Leu107Ser in COX1 and Gly33Glu in COX3 are located close to each other (distance of approximately 9 Å) on the surface of the protein structures, making them available targets for binding with deltamethrin. Moreover, deltamethrin exposure caused

behavioural toxicity and whole-body ATP depletion in drug susceptible *L. salmonis*, but not resistant lice (Carmona-Antoñanzas et al., 2017). In addition, deltamethrin exposure affected transcript expression of mitochondrial genes and increased apoptosis in subcuticular tissue, skeletal muscle and central ganglion cells in drug susceptible lice, with such effects being less pronounced in deltamethrin resistant parasites (Bakke et al., 2018). However, further research is needed to pinpoint potential mitochondrial targets for deltamethrin toxicity in salmon lice and to identify the molecular mechanism by which mtDNA mutations confer deltamethrin resistance.

1.10.2 Target site mutations in voltage gated sodium channels

As described in section 1.10.1, Carmona-Antoñanzas et al. (2017) crossed deltamethrin resistant males with drug susceptible females and in some families 20% of the F2 progenies were resistant, indicating a contribution of nuclear genes in the resistance phenotype (Fig. 1.10).

In terrestrial arthropods, a common mechanism of pyrethroid resistance is *kdr*-resistance, which results from nuclear non-synonymous point mutations in Na_v1. In *L. salmonis*, three homologues of Na_v1 have been identified, which are named $LsNa_v1.1$, $LsNa_v1.2$ and $LsNa_v1.3$ (Carmona-Antoñanzas et al., 2019). Characterisation of the three Na_v homologues led to the identification of one putative *kdr* mutation in DII S5 of $LsNa_v1.3$ (Carmona-Antoñanzas et al., 2019), which causes an amino acid change (I936V, numbering according to *M. domestica* Na_v1; NCBI accession no. AAB47604) in the predicted pyrethroid binding site of the channel and is homologous to a previously described *kdr* mutation in pyrethroid-resistant isolates of *H. zea* (Hopkins and Pietrantonio, 2010; Usherwood et al., 2007).

Another putative *kdr* mutation was described by Fallang et al. (2005). The authors identified a non-synonymous mutation (Q945R, numbering according to *M. domestica* Na_v1; NCBI accession no. CAA65448.1) in DII S5 of $LsNa_v1.1$ in 10 to 67% of all partially sequenced *L. salmonis* from farm sites with reduced treatment efficacy of pyrethroids. However, analyses were restricted to DII and the salmon lice were not individually selected for resistance. Neither Carmona-Antoñanzas et al. (2019) nor Helgesen (2015) found *kdr*-type mutations in both $LsNa_v1.1$ and $LsNa_v1.2$ from individually selected pyrethroid resistant salmon lice from Scotland and Norway.

1.10.3 Increased detoxification

1.10.3.1 Cytochrome P450 monooxygenases

In bioassay experiments pre-treatment with the monooxygenase inhibitor piperonyl butoxide increased the sensitivity of *L. salmonis* towards both deltamethrin and cypermethrin (Sevatdal et al., 2005b). In addition, a positive but not statistically significant correlation was found between the peroxidase activity in haem-containing enzymes, which include CYPs, and EC_{50} values for deltamethrin and cypermethrin. The authors concluded that CYPs may be involved in deltamethrin detoxification in salmon lice. In a microarray study with copepodid *L. salmonis*, cypermethrin exposure induced overexpression of three CYPs (Poley et al., 2016).

More than a decade later, the CYP superfamily of *L. salmonis* was annotated and systematically assessed regarding its potential roles in drug resistance (Humble et al., 2019). The authors identified 25 CYP genes in *L. salmonis*, making it the smallest CYP superfamily of all arthropods characterised to date. CYP transcript expression did not differ between one drug susceptible and one multi-resistant strain of *L. salmonis*, but exposure to deltamethrin induced significant transcriptional upregulation of a few CYPs (CYP3041E2, CYP3027H3, CYP3649A1) in resistant *L. salmonis* compared to untreated control animals. However, this data does not provide systematic evidence for a role of CYP genes in mediating deltamethrin resistance in salmon lice (Humble et al., 2019).

1.10.3.2 Serine hydrolases

In *L. salmonis*, no correlation was found between production of unspecific esterase and pyrethroid resistance (Sevatdal et al., 2005b). However, the CaE family of *L. salmonis* has not yet been annotated and systematically assessed regarding its potential roles in drug resistance.

Ester bonds within pyrethroids can also be hydrolysed by certain proteases with a catalytic triad of Ser, His, and Asp such as trypsin and chymotrypsin (Perona and Craik, 1995). Caligid sea lice have been shown to express high levels of proteases, which are involved in several physiological processes including yolk degradation and utilisation in larvae, attachment to the host, and digestion of the host's tissue (Fast et al., 2007; Skern-Mauritzen et al., 2009). In *C. rogercresseyi*, 44 putative trypsin-like and seven putative chymotrypsin-like transcripts showed differentiated transcriptional modulation in response to 3 µg L^{-1} deltamethrin (Valenzuela-Miranda and Gallardo-Escárate, 2016), and in a microarray study with *L. salmonis*, cypermethrin exposure induced overexpression of five trypsin-like proteases (Poley et al., 2016).

1.10.3.3 Glutathione-S-transferases and antioxidant enzymes

In *C. rogercresseyi*, transcriptional activity of GSTs remained unchanged after exposure to 1 to 3 µg L^{-1} deltamethrin (Chavez-Mardones and Gallardo-Escárate, 2014), but transcription of SOD, catalase, peroxiredoxin, and phospholipid-hyperoxide glutathione peroxidase was significantly upregulated following exposure to 2 µg L^{-1} deltamethrin. In a microarray study with copepodid *L. salmonis*, cypermethrin exposure did not induce differential expression of GSTs, SODs, catalase, and peroxidases (Poley et al., 2016).

1.10.3.4 ABC-transporters

In *C. rogercresseyi*, 57 full or partial genes encoding ABC-transporter proteins were identified and the complete cDNA of one *C. rogercresseyi* P-glycoprotein (P-gp) gene, Cr-Pgp, has been isolated (NCBI accession number KF704367) (Valenzuela-Muñoz et al., 2015, 2014). Following treatment with 3 ppb deltamethrin, ABC-transporter subfamilies D, E, and F were highly regulated in adult lice (Valenzuela-Muñoz et al., 2015). Moreover, exposure to 2 µg L^{-1} deltamethrin increased expression levels of Cr-Pgp in females (Valenzuela-Muñoz et al., 2014).

In *L. salmonis*, 33 genes encoding ABC-transporter proteins were identified, and 18 sequences were assigned to subfamilies containing drug transporter proteins (Carmona-Antoñanzas et al., 2015). To date, seven of these ATP-transporters have been cloned and studied, including three P-gp (Heumann et al., 2014, 2012; Tribble et al., 2007). In *L. salmonis*, the involvement of ABC-transporters in deltamethrin resistance has not yet been investigated.

1.10.4 Reduced cuticular penetration

In *L. salmonis*, ^{14}C-labelled deltamethrin is mainly taken up through the cuticle of the ventral surface and subsequently transported via the haemolymph throughout the body (Sevatdal et al., 2005b). Accordingly, cuticle thickening or alterations of the cuticle composition may reduce the penetration of pesticides through the cuticle (reviewed by Balabanidou et al., 2018), and thus may contribute to pyrethroid resistance in caligid sea lice. However, to date only one preliminary study investigated the uptake of ^{14}C-deltamethrin in resistant and susceptible *L. salmonis* (Bakke et al., 2016). In that experiment, no difference in cuticular penetration was found between resistant and susceptible lice directly after exposure to ^{14}C-deltamethrin.

Objectives

The overarching aim of this PhD thesis is to identify molecular determinants for deltamethrin resistance in *L. salmonis* and to obtain insights into the underlying mechanisms of resistance. Outcomes from this study are expected to contribute to the improvement of treatment strategies targeting, particularly the optimisation of resistance monitoring and management.

To achieve this aim, this PhD thesis will investigate the relative contribution of Na_v1 target-site mutations and mtDNA mutations in deltamethrin resistance in salmon lice, both of which have been suggested previously (Carmona-Antoñanzas et al., 2019, 2017). In addition, the potential involvement of the CaE gene family will be examined, which has been shown to contribute to pyrethroid resistance in several terrestrial arthropods (reviewed by Oakeshott et al., 2005).

The following hypotheses will be tested:

1. Deltamethrin resistance in *L. salmonis* involves mutations of the pyrethroid target-site, the voltage gated sodium channel, and can be predicted based on parasite genotypes at a previously reported SNP locus in the channel subunit $Na_v1.3$.
2. Deltamethrin resistance in *L. salmonis* involves mutations of the mtDNA and can be predicted based on parasite genotypes at previously reported mtDNA SNP loci.
3. Deltamethrin resistance of *L. salmonis* involves detoxification and/or sequestration by CaEs.

This PhD thesis is composed of three chapters, which summarise experiments conducted to test the above hypotheses.

The first chapter aims to investigate the relative importance of Na_v1 target-site mutations as compared to mtDNA mutations as determinants of deltamethrin resistance in L. salmonis.

To obtain insights into marker association with deltamethrin resistance, deltamethrin susceptible and resistant *L. salmonis* will be genotyped for a $Na_v1.3$ mutation and selected mitochondrial SNPs, which were associated with deltamethrin resistance in previous studies (Carmona-Antoñanzas et al., 2017; Carmona-Antoñanzas et al., 2018). Samples tested will include *L. salmonis* of deltamethrin resistant and susceptible laboratory strains, *L. salmonis* collected from Scottish production sites and phenotyped by bioassays, as well as archived *L. salmonis* derived from a family obtained by crossing a deltamethrin resistant male and a deltamethrin susceptible female. Genetic tests will be complemented by bioassays with the non-ester pyrethroid etofenprox, a compound that has been used to detect target-site resistance based on Na_v1 mutations.

The second chapter aims to differentiate mtDNA SNPs that are potentially causally linked to deltamethrin resistance from other hitchhiking SNPs.

L. salmonis will be obtained from a range of Scottish farm sites and rated as deltamethrin resistant or susceptible based on bioassays before being subjected to genotyping at selected mitochondrial SNP loci. Mitochondrial haplotypes will be established and haplotype association with deltamethrin resistance assessed. One resistant haplotype distinct from the resistant haplotype characterised in a previous study will be selected for further studies (Carmona-Antoñanzas et al., 2017), which will involve assessing the mode of inheritance of deltamethrin resistance conferred by the haplotype, and its susceptibility to ATP depletion during deltamethrin exposure. Comparing the mtDNA sequence between well-characterised resistant and susceptible *L. salmonis* strains is expected to generate new hypotheses about the involvement of specific mitochondrial SNPs in the resistance mechanism.

The third chapter aims to identify members of the CaE family in L. salmonis and characterise their potential roles in resistance of the parasite to deltamethrin.

Sequences encoding *L. salmonis* CaEs will be identified by homology searches of transcriptome and genome assemblies and annotated. CaE sequences will then be analysed *in silico* to identify CaEs that are predicted to be catalytically competent and thus may potentially contribute to pesticide resistance. CaEs predicted to be catalytically active will be characterised regarding their cDNA sequence and their transcript expression in laboratory-maintained strains of multi-resistant and drug-susceptible *L. salmonis*, and effects of deltamethrin exposure on CaE transcript expression will be determined. In addition, SNPs of CaEs predicted to be catalytically competent will be assessed in *L. salmonis* strains differing in deltamethrin susceptibility.

Chapter 2
Investigation of deltamethrin resistance in salmon lice (*Lepeophtheirus salmonis*) provides no evidence for roles of mutations in voltage-gated sodium channels

2.1 Abstract

The pyrethroid deltamethrin is used to treat infestations of farmed salmon by parasitic salmon lice, *Lepeophtheirus salmonis*. However, the efficacy of deltamethrin for salmon delousing is threatened by resistance development. In terrestrial arthropods, *kdr* mutations of the Na_v, the molecular target for pyrethroids, can cause deltamethrin resistance. A putative *kdr* mutation of an *L. salmonis* sodium channel homologue (LsNa$_v$1.3 I936V) has previously been identified. At the same time, deltamethrin resistance of *L. salmonis* has been shown to be inherited maternally and to be associated with mtDNA mutations. The present study assessed potential roles of the above putative *kdr* mutation as a determinant of deltamethrin resistance in laboratory strains and field populations of *L. salmonis*.

The deltamethrin resistant *L. salmonis* strain IoA-02 expresses the LsNa$_v$1.3 I936V mutation but was susceptible to the non-ester pyrethroid etofenprox, a compound against which pyrethroid resistant arthropods are usually cross-resistant if resistance is caused by Na_v mutations. In a family derived from a cross between an IoA-02 male and a drug-susceptible female lacking the *kdr* mutation, deltamethrin resistance was not associated with the genotype at the LsNa$_v$1.3 locus ($P > 0.05$). Similarly, in Scottish field populations of *L. salmonis*, LsNa$_v$1.3 I936V showed no association with deltamethrin resistance. In contrast, genotypes at the mtDNA loci A14013G and A9030G were significantly associated with deltamethrin resistance ($P < 0.001$).

In the studied *L. salmonis* isolates, deltamethrin resistance was unrelated to the LsNa$_v$1.3 I936V mutation but showed close association with mtDNA mutations.

2.2 Introduction

Sea lice of the family Caligidae (Copepoda) are ectoparasites infecting farmed and wild marine fish, feeding on the mucus, skin, and blood of the host (Boxaspen, 2006). When reaching high severity, caligid infections can cause skin lesions associated with a high risk of secondary infections, osmoregulatory dysfunction, immunosuppression, increased stress, and reduced food conversion and growth rates (Grimnes and Jakobsen, 1996; Wootten et al., 1982). In 2018, the estimated global costs of sea lice infections to the salmon industry were approximately USD $873 million/£700 million (Brooker et al., 2018b), comprising mainly the costs for treatments and to a lesser extent losses in production. In the Northern hemisphere, most sea lice infections of salmonid fish are caused by the salmon louse *Lepeophtheirus salmonis*

(Aaen et al., 2015). At salmon production sites, sea lice are controlled by integrated pest management strategies combining non-medicinal approaches, such as mechanical and thermal delousing, physical barriers (Holan et al., 2017), and biological control through co-culture with cleaner fish (Brooker et al., 2018a), and medicinal approaches employing a limited range of licensed veterinary medicines (Overton et al., 2019). Pharmaceuticals used for salmon delousing agents are administered as medicated feeds or topical bath treatments. Oral treatments include the macrocyclic lactone emamectin benzoate and different benzoylureas, while bath treatments include the organophosphate azamethiphos, the disinfectant hydrogen peroxide and the pyrethroids cypermethrin and deltamethrin (Helgesen et al., 2019). However, in *L. salmonis* populations of the North Atlantic, loss of efficacy has been reported for most available salmon delousing agents (Fjørtoft et al., 2020, 2017; Helgesen et al., 2019).

Pyrethroids, which are synthetic analogues of the botanical pyrethrins (Soderlund, 2012), are widely used to control insects that are phytophagous, parasitic, or represent vectors for human disease (Zhang, 2018). In 2014, pyrethroids accounted for 17% of global insecticide use (Zhang, 2018). In arthropods, the toxic action of pyrethroids is based on their blocking of Na_v, which plays an essential role in the initiation and propagation of nerve impulses (Lowenstein, 1942). In terrestrial arthropods, two main mechanisms of deltamethrin resistance are known: *Kdr* resistance by target-site mutations in Na_v (Knipple et al., 1994; Williamson et al., 1993), and increased detoxification by enhanced expression of metabolic enzymes, such as CaEs, CYPs, or GSTs (Ranson et al., 2002).

In *L. salmonis*, resistance to the pyrethroid deltamethrin is widespread (Fjørtoft et al., 2020), but its molecular mechanisms remain to be resolved. Recently, two types of genetic determinants for pyrethroid resistance in *L. salmonis* have been suggested. On the one hand, the characterisation of three Na_v homologues in *L. salmonis*, $LsNa_v1.1$, $LsNa_v1.2$ and $LsNa_v1.3$, led to the identification of a putative *kdr* mutation in $LsNa_v1.3$ (Carmona-Antoñanzas et al., 2018), which causes an amino acid change (I936V) in the predicted pyrethroid binding site of the channel and is homologous to a previously described *kdr* mutation in pyrethroid-resistant isolates of the phytophagous moth *H. zea* (Hopkins and Pietrantonio, 2010; Usherwood et al., 2007). On the other hand, crossing experiments in which deltamethrin resistant *L. salmonis* were interbred with drug susceptible parasites of the opposite sex to generate multigenerational families revealed a predominantly maternal inheritance of deltamethrin resistance (Carmona-Antoñanzas et al., 2017). In particular, all F2 parasites of families derived from crosses between resistant females and susceptible males were resistant, whereas <20% resistant F2 parasites were observed in the inverse crosses derived from susceptible females and resistant males, suggesting a role of mitochondrial genes as determinants of deltamethrin resistance

(Carmona-Antoñanzas et al., 2017). Deltamethrin resistance in independent isolates obtained from different Scottish regions was associated with virtually identical mitochondrial (mtDNA) haplotypes, which contained the mitochondrial SNP A14017G located within the CytB gene and SNP A9030G located in cytochrome c oxidase subunit 1 (COX1) gene (Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015). These findings suggested that the mode of action of deltamethrin in *L. salmonis* might involve mitochondrial targets. In support of this hypothesis, deltamethrin has been shown to affect mitochondrial functions such as ATP production (Carmona-Antoñanzas et al., 2017) and induced apoptosis in skeletal muscle tissues, which have high mitochondria content (Bakke et al., 2018). However, efforts to disentangle the role of nuclear and mitochondrial mutations in pyrethroid resistance are complicated by the fact that deltamethrin resistant *L. salmonis* investigated in previous studies displayed both the putative *kdr* mutation $LsNa_v1.3$ I936V and mtDNA mutations (Carmona-Antoñanzas et al., 2017; Carmona-Antoñanzas et al., 2018).

The aim of the present study was to assess potential roles of the $LsNa_v1.3$ I936V mutation as a determinant of deltamethrin resistance in *L. salmonis*. First, *L. salmonis* strains differing in deltamethrin resistance and expression of the $LsNa_v1.3$ I936V mutation were investigated regarding their susceptibility to a non-ester pyrethroid, that is a type of compound towards which pyrethroid resistant parasites can be expected to be cross-resistant if the resistance mechanism is based on *kdr*-type mutations. Second, selected archived samples of the above previously published crossing experiment, for which deltamethrin bioassay data were available, were studied. F2 parasites of a family derived from a deltamethrin resistant male and a drug-susceptible female *L. salmonis* were genotyped to assess the potential association of the LsNav1.3 I936V mutation with resistance in the absence of interference by potential resistance-associated mtDNA haplotypes. Finally, *L. salmonis* were collected from a range of Scottish farm sites. Parasites rated as deltamethrin resistant or susceptible in bioassays were subjected to genotyping at the above loci to obtain insights into marker association with deltamethrin resistance in field populations.

2.3 Materials and methods

2.3.1 Ethics statement

All research projects involving the University of Stirling (UoS) are subject to a thorough Ethical Review Process prior to any work being approved. The present research was assessed by the UoS Animal Welfare Ethical Review Body (AWERB) and passed the ethical review process. Laboratory infections of Atlantic salmon with *L. salmonis* were performed under a valid UK Home Office licence and at low parasite densities unlikely to compromise fish welfare.

2.3.2 *Lepeophtheirus salmonis* strains and husbandry

Laboratory *L. salmonis* strains used in this study have been described in detail elsewhere (Carmona-Antoñanzas et al., 2016; Heumann et al., 2012). Strain IoA-00, which was taken into culture in 2003, is susceptible to all current salmon-delousing agents. Strain IoA-02 was established in 2011 and is resistant to deltamethrin. Deltamethrin median effective concentrations (EC_{50}) of both strains have been determined previously (IoA-00: 0.28 µg L^{-1} (95% confidence limits: 0.23-0.36 µg L^{-1}; based on 30 min exposure followed by 24h recovery in seawater); IoA-02 40.1 µg L^{-1} (22.1-158.9 µg L^{-1}; 30 min exposure and 24h recovery in seawater) (Carmona-Antoñanzas et al., 2017).

L. salmonis strains were maintained without further pesticide selection under standardised conditions at the Marine Environmental Research Laboratory of the University of Stirling (Machrihanish, UK), as described in detail elsewhere (Heumann et al., 2012). In brief, *L. salmonis* were kept on Atlantic salmon held in circular tanks provided with a continuous supply of seawater at ambient temperature and a photoperiod corresponding to natural day length. To propagate *L. salmonis* lines, egg strings obtained from gravid females were hatched and incubated to the infective copepodid stage, which was used to infect naïve Atlantic salmon. Infection trials were set up to produce preadult II and adult parasites for bioassays and molecular analyses. Host fish were euthanised using a UK Home Office approved Schedule 1 method prior to the removal of salmon lice from fish.

2.3.3 *Lepeophtheirus salmonis* crosses

This study included molecular analyses of archived *L. salmonis* siblings of one family from a previously published crossing experiment (Fig. S2.1), with the detailed procedures of performing the cross having been reported elsewhere (Carmona-Antoñanzas et al., 2017). In brief, the family was established at the P0 level by crossing a deltamethrin resistant male (IoA-02 strain) and a deltamethrin susceptible female (IoA-00 strain). The offspring of this cross was obtained and grown out to appropriate life stages, allowing the setting up of three breeding sibling pairs of F1 parasites in new tanks and production of another generation (F2), which was allowed to develop

to the male adult/female preadult II stages. F2 parasites were then subjected to bioassays to determine the deltamethrin susceptibility phenotype of each individual (see section 2.3.5).

2.3.4 *Lepeophtheirus salmonis* field populations

Bioassays and molecular analyses were carried out with *L. salmonis* obtained from five Scottish aquaculture sites from Sutherland (2017, N = 132; 2019, N = 180), Argyll and Bute (2018, N = 86; 2019, N = 220), and Inverness-shire (2019, N = 180) (Table S2.1). Lice were collected during weekly lice counts and routine veterinary procedures, placed in plastic bags containing cool (12°C) aerated seawater and shipped to the laboratory for bioassay (see section 2.3.5). Samples were transported in insulated boxes equipped with cold packs and arrived within 6 h of initial collection.

2.3.5 *Lepeophtheirus salmonis* bioassays

Bioassays were performed to assess the susceptibility of salmon lice to deltamethrin and the non-ester pyrethroid etofenprox. Deltamethrin and etofenprox (Pestanal® analytical standard grade) were purchased from Sigma-Aldrich (Gillingham, UK). A subset of deltamethrin bioassays was performed using AlphaMax® (Oslo, PHARMAQ AS, 10 mg deltamethrin mL^{-1}). *L. salmonis* adult males and preadult-II females used in bioassays were collected from host fish, transported to the laboratory as described above, and maintained overnight in aerated seawater at 12°C. To set-up bioassays, individual parasites displaying normal attachment and swimming behaviour were randomly allocated to 300 mL crystallising dishes containing 100 mL of filtered (55 µm) seawater, with each dish receiving 5 preadult-II females and 5 adult males (selected to provide similar sizes). All bioassay incubations took place in a temperature-controlled chamber set to 12°C. Chemical exposures were initiated by adding 50 µL of a 2000x final concentration solution of the relevant compound to glass crystallising dishes containing 100 mL seawater and parasites. To prepare the 2000x final concentration solutions, etofenprox was solubilised in ethanol, whereas deltamethrin was dissolved in polyethylene glycol (PEG_{300}, M_n = 300) or acetone. The final solvent concentration was 0.05% (v/v) in all tests.

Bioassays performed in this study had either a standard or a single-dose design (Helgesen and Horsberg, 2013a; Sevatdal and Horsberg, 2003). In standard bioassays, salmon lice were exposed to different concentrations of the tested compound (deltamethrin: four to six concentrations in the range of 0.1 and 20 µg L^{-1}; etofenprox: 0.05, 0.1, 0.22, 0.46, 1, 2.15, 4.62 µg L^{-1}). The design further comprised a solvent control. Duplicate test dishes were included for each chemical and control treatment. Single-dose bioassays, which allow determination of the susceptibility phenotype of individual parasites (see below), were conducted in an analogous fashion, except that deltamethrin was provided at one diagnostic concentration (2 µg L^{-1}). In deltamethrin

bioassays, salmon lice were exposed to the compound for 30 min and then allowed to recover in clean seawater for 24 h prior to behavioural responses being examined and rated. In bioassays with etofenprox, chemical exposure was for 24 h, directly followed by examination and rating of test animals. After the completion of bioassays, lice were stored in absolute ethanol at -20°C pending DNA extraction and genetic analyses.

Rating criteria based on observed behavioural responses have been described in detail elsewhere (Carmona-Antoñanzas et al., 2016). Parasites rated as "live" or "weak" were considered unaffected, while "moribund" and "dead" parasites were considered affected. Bioassays were considered invalid if the number of affected lice in solvent controls exceeded 10%. In standard bioassays, the susceptibility of the tested population was characterised by probit analysis (see section 2.3.8), whereas in single-dose bioassays involving exposure to 2 µg L^{-1} deltamethrin parasites were classified as deltamethrin resistant if they were rated unaffected and susceptible if rated affected at the completion of bioassays.

2.3.6 DNA extraction

Genomic DNA was extracted from individual ethanol-conserved salmon louse specimens using a high-throughput protocol (Montero-Pau et al., 2008). A small piece (~2 mm) of the cephalothorax was cut off and transferred into a 0.2 mL tube containing 100 µL alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA, pH 12.0). Samples were heated to 95°C for 30 min and subsequently cooled to 4°C for 5 min using a polymerase chain reaction (PCR) thermocycler. Then, 100 µL 40 mM Tris-HCL (pH 5.0) was added, and samples vortexed briefly before being centrifuged at 4000xg for 1 min. Crude DNA extracts were stored at -20°C pending use in genotyping analyses.

2.3.7 Genotyping of single nucleotide polymorphism (SNP) alleles

PCR based genotyping assays employing universal fluorescence energy transfer (FRET) probes (KASP® 4.0, LGC Genomics, Teddington, UK) were designed to detect $LsNa_v1.3$ SNP A3041G, corresponding to I936V in *M. domestica* Na_v1 (Numbering according to NCBI accession number AAB47604) (Carmona-Antoñanzas et al., 2018), and *L. salmonis* mtDNA SNPs A14013G (CytB, cds) and A9030G (COX1, cds; Numbering according to NCBI accession number LT630766.1) (Carmona-Antoñanzas et al., 2017), with oligonucleotide primers shown in Table S2.2. Each sample was genotyped in duplicate 10 µL reactions containing 1 µL DNA extract, 0.14 µL KASP® Assay Mix and 5 µL 2x KASP® Master Mix (LGC genomics, Teddington, UK). Each assay run also included non-template controls, in which extraction buffer replaced the DNA sample. Reactions were set up in 96-well plates and subjected to the following thermocycling programme: Activation (94°C for 15 min), then ten touch-down cycles (denaturation at 94°C for 20 s,

annealing at 65-57°C (dropping 0.8°C per cycle) for 60 s), followed by 35 cycles (94°C for 20 s and 57°C for 60 s). *L. salmonis* genotypes were assigned after reading the fluorescence emission of the allele specific FAM and HEX fluorophores for each sample using endpoint genotyping software and the Quantica PCR thermal cycler (Bibby Scientific, Stone, UK).

Afterwards, the classification success for deltamethrin sensitivity (%) was determined for the mtDNA markers A14013G and A9030G, and the Na_v marker A3041G. The classification success of each SNP marker was calculated based on the SNP genotype determined by PCR based genotyping assays compared with the resistance phenotype interfered from single-dose bioassays involving exposure to 2 µg L^{-1} deltamethrin. The classification success comprises of both the compliance of the wild type (WT) SNP genotype with deltamethrin susceptibility, and the mutant (Mu) SNP genotype with deltamethrin resistance.

$$\text{Classification success (\%)} = 100 * \left(\frac{n_{WT\ genotype} * \text{Proportion susceptible (within WT)} + n_{Mu\ genotype} * \text{Proportion resistant (within Mu)}}{n_{WT\ genotype} + n_{Mu\ genotype}} \right)$$

WT: Wild type. Mu: Mutant. Proportion susceptible (within WT): Number of phenotypic susceptible individuals with WT genotype divided by total number of individuals with WT genotype. Proportion resistant (within Mu): Number of phenotypic resistant individuals with Mu genotype divided by total number of individuals with MU genotype.

For the mtDNA markers, individuals were either WT (allele A) or Mu (allele G), with WT animals predicted to be susceptible and Mu lice resistant. For the Na_v marker A3041G, individuals were homozygous WT (A/A), homozygous Mu (G/G), or heterozygous (A/G). The classification success of this marker was calculated in two ways, assuming either recessive inheritance of resistance related to the Na_v mutation, or an incomplete recessive mode of inheritance (Stone, 1968). Assuming recessive inheritance, phenotypic resistance was predicted for Mu homozygous individuals, whereas homozygous WT and heterozygous lice were predicted to be susceptible. For the alternative case of incomplete recessive inheritance, it was assumed that the mutation's effects are sufficient to result in both Mu homozygous and heterozygous individuals to remain unaffected by the diagnostic deltamethrin concentration employed in the bioassay, with these animals being predicted to be resistant and homozygous WT animals to be susceptible.

2.3.8 Data analyses and statistical tests

The concentration-response relationship for compounds tested in *L. salmonis* bioassays was assessed by probit analysis using the statistical program R version 3.6.0 (package *drc*), assuming a log-normal distribution of drug susceptibility. Based on the fitted models, EC_{50} and 95% confidence limits were derived and effects of sex and strain on drug susceptibility tested. Genotype and allele frequencies for SNPs A3041G (LsNa$_v$1.3), A9030G (COX1), and A14013G

(CytB) were compared between resistant and susceptible individuals of the same population using Fisher's exact probability test, as implemented in the program Genepop version 4.2 (https://genepop.curtin.edu.au/). The significance level was set at $P < 0.05$.

2.4 Results

2.4.1 Susceptibility of *L. salmonis* strains to etofenprox

L. salmonis strains included in this study have been characterised previously. Strain IoA-00 is drug susceptible, whereas strain IoA-02 is 143-fold resistant to deltamethrin (Carmona Antoñanzas et al., 2017) and further shows hyposensitivity to emamectin benzoate and azamethiphos (Heumann et al., 2012; Humble et al., 2019). Strain IoA-02 shows a high allele frequency of the putative *kdr* mutation LsNa$_v$1.3 A3041G, while all tested individuals of the IoA-00 strain show the wild type allele at this locus (Table 2.1). IoA-00 and IoA-02 showed similar susceptibility to the non-ester pyrethroid etofenprox (Table 2.2).

Table 2.1 Genetic association of nuclear and mitochondrial single nucleotide polymorphisms (SNPs) with deltamethrin resistance in *L. salmonis* laboratory strains and their F2 progenies. Male salmon lice from laboratory strain IoA-02 and female lice from strain IoA-00 were crossed to produce families spanning one parental and two filial generations (F1, F2) (Fig. S2.1). Deltamethrin susceptible and resistant individuals from strains IoA-00 and IoA-02, and F2 progenies were subjected to allele specific PCR genotyping at nuclear SNP A3041G (voltage-gated sodium channel homologue LsNa$_v$1.3) and mitochondrial SNPs A14013G (cytochrome B; CytB) and A9030G (cytochrome c oxidase subunit 1; COX1).

		Laboratory strains		**F2 generation derived from cross**	
		IoA-00	IoA-02	IoA-02 male x IoA-00 female	
		Susceptible†	**Resistant**†	**Susceptible**‡	**Resistant**‡
n		14	16	55	13
LsNa$_v$1.3 A3041G					
Genotype frequencies	**A/A**	1.0	0	0.40	0.23
	A/G	0	0.06	0.56	0.69
	G/G	0	0.94	0.04	0.08
Allele frequency	**G**	0	0.97	0.32	0.42
Genotypic/allelic differentiation *P*-value Fisher's exact test		<0.001/<0.001		0.27/0.36	
Mitochondrial SNPs A14013G (CytB) or A9030G (COX1)					
Allele frequencies	**A**	1.0	0	1.0	1.0
	G	0	1.0	0	0

†Deltamethrin susceptibility of parasites is assumed to correspond to the drug susceptibility of their strains of origin, with IoA-02 individuals being considered resistant and IoA-00 individuals being considered susceptible.

‡Deltamethrin susceptibility of F2 progenies was determined in single-dose bioassays, involving exposure (30 min) to 2 µg l^{-1} deltamethrin, followed by recovery in seawater (24 h) and subsequent rating as susceptible (affected) or resistant (unaffected).

Table 2.2 Susceptibility of laboratory *L. salmonis* strains to etofenprox. Bioassays involved 24 h exposure of salmon lice of the deltamethrin susceptible laboratory strain IoA-00 and the deltamethrin resistant strain IoA-02 to etofenprox, followed by rating of lice as normal or affected. Medium effective concentrations (EC_{50}) were derived by probit analyses. Gender and strain differences in drug susceptibility were assessed by comparing probit models of dose-response relationships. Values with different letters for each pesticide are significantly different ($P < 0.05$).

L. salmonis strain	EC_{50} (µg L^{-1}) and 95% confidence limits	
	Female	**Male**
IoA-00	0.42 (0.29-0.54)[c, e]	0.32 (0.23-0.40)[d, e]
IoA-02	0.55 (0.38-0.72)[c]	0.26 (0.19-0.32)[d]
Resistance ratio[†]	**1.31**	**0.81**

[†]Resistance ratio of etofenprox: EC_{50} IoA-02/EC_{50} IoA-00.

2.4.2 Association of *L. salmonis* SNP alleles with deltamethrin resistance in a crossing experiment

A crossing experiment between the deltamethrin resistant *L. salmonis* strain IoA-02 and the drug-susceptible strain IoA-00 has been reported previously (Carmona-Antoñanzas et al., 2017). In a family derived from an IoA-00 female and an IoA-02 male, 20% of F2 parasites were resistant (Fig. S2.1) (Carmona-Antoñanzas et al., 2017). In the present study, available archived F2 specimen of the experiment, as well as parental strain *L. salmonis*, were genotyped at the SNP loci LsNa$_v$1.3 A3041G and mtDNA A14013G (CytB) and A9030G (COX1) (Table 2.1). Confirming earlier reports (Carmona-Antoñanzas et al., 2017), allele frequencies at both loci differed significantly ($P < 0.001$) between the two parental strains, with IoA-02 showing fixation for the mtDNA mutations. As expected, F2 parasites showed the same mtDNA genotypes as their IoA-00 grandmother, while at the LsNa$_v$1.3 SNP A3041G locus, all conceivable genotypes were observed in F2 animals. Genotype and allele frequencies of LsNa$_v$1.3 SNP A3041G did not differ between deltamethrin resistant and deltamethrin susceptible F2 individuals ($P > 0.05$).

2.4.3 Deltamethrin resistance in field populations of *L. salmonis*

The deltamethrin susceptibility of *L. salmonis* populations was determined for Scottish field sites sampled between 2017 and 2019. Deltamethrin EC_{50} values ranged from 1.6 to 8.0 µg L^{-1}, demonstrating reduced susceptibility for all tested populations (Table 2.3). The deltamethrin susceptibility of salmon lice from three Scottish field sites was further characterised individually as susceptible or resistant, based on their behavioural response at 2 µg L^{-1} deltamethrin. When deltamethrin resistance was assessed based on parasite responses at a diagnostic deltamethrin concentration (2 µg L^{-1}), 67 to 94% of parasites were found to be deltamethrin resistant (Table 2.4).

Table 2.3 Susceptibility of *L. salmonis* from different Scottish aquaculture production sites to deltamethrin. Bioassays involved exposure (30 min) to deltamethrin, followed by recovery in seawater (24 h) and rating of lice as normal or affected. Median effective concentrations (EC_{50}) were derived by probit analyses.

Scottish county	Year	EC_{50} (µg L^{-1})† and 95% confidence limits
Sutherland 17	2017	1.60 (0.91-2.29)
Argyll 18	2018	>2.0‡
Argyll 19	2019	8.00 (5.74-10.23)
Sutherland 19	2019	2.49 (1.63-3.36)
Inverness 19	2019	5.4 (4.1-7.2)

†Raw data used to determine median effective concentrations (EC_{50}) are provided in Table S2.1.
‡Single-dose bioassay: 94.4% (N=54) remained unaffected after exposure to 2 µg L^{-1} deltamethrin.

Table 2.4 Genetic association of single nucleotide polymorphisms A3041G in voltage-gated sodium channel homologue $LsNa_v1.3$ with deltamethrin (DTM) resistance in *L. salmonis*. Salmon lice from Scottish aquaculture sites were classified as DM susceptible (S) or resistant (R) based on bioassays. Susceptible and resistant individuals were subjected to allele specific PCR genotyping at SNP A3041G. CS: Classification success of A3041G for DTM sensitivity.

Origin of *L. salmonis*	DTM resistance†	n	Genotype frequencies			G Allele	*P*-value‡ Genotypic/ allelic differentiation	CS (%) for recessive inheritance of resistance trait§	CS (%) for incomplete recessive inheritance of resistance trait§
			A/A	A/G	G/G				
Argyll 18	S	3	0.33	0.33	0.33	0.50	0.65/0.67	13	63
	R	51	0.35	0.55	0.1	0.37			
Argyll 19	S	7	0.43	0.57	0.00	0.29	0.78/1.0	39	46
	R	21‡	0.52	0.29	0.19	0.33			
Sutherland 19	S	10	0.70	0.30	0	0.15	0.70/0.74	33	50
	R	20	0.60	0.40	0	0.20			
Total	**S**	**20**	**0.55**	**0.40**	**0.05**	**0.25**	**0.45/0.35**	**25**	**55**
	R	**92**	**0.45**	**0.46**	**0.10**	**0.31**			

†Susceptibility to deltamethrin was determined in single-dose bioassays, involving exposure (30 min) to 2 µg L^{-1} deltamethrin, followed by recovery in seawater (24 h) and subsequent rating as susceptible (affected) or resistant (unaffected).
‡21 out of 37 deltamethrin resistant individuals were subjected to allele specific PCR genotyping at SNP A3041G.
§Reflecting recessive inheritance, phenotypic resistance was predicted by mutant homozygous (G/G) individuals. Reflecting incomplete recessive inheritance, phenotypic resistance was predicted by both mutant homozygous (G/G) and heterozygous (A/G) individuals.

2.4.4 Association of nuclear and mtDNA SNP alleles with deltamethrin resistance in field populations

Genotype and allele frequencies of the LsNa$_v$1.3 SNP A3041G did not differ ($P > 0.05$) between deltamethrin resistant and susceptible salmon lice from field populations (Table 2.4). Na$_v$ target-site resistance has been shown to be inherited either as a recessive or incomplete recessive trait (Brito et al., 2013; Chang et al., 2012; Farnham, 1977; Martinez-Torres et al., 1998; Sun et al., 2016), depending on the investigated species and laboratory strain. Accordingly, the classification success of Na$_v$ marker A3041G was calculated for both modes of inheritance. However, regardless of the calculation approach, the SNP genotype did not comply with phenotypic resistance in bioassays. In contrast, allele frequencies of both mtDNA SNPs A14013G (CytB) and A9030G (COX1) differed significantly ($P < 0.001$) between all tested deltamethrin resistant and susceptible lice from Scottish field sites (Table 2.5). Genotypic classification of deltamethrin resistance based on mtDNA markers A14013G and A9030G showed a 79% compliance with phenotypic classification based on bioassays.

Table 2.5 Genetic association of mitochondrial single nucleotide polymorphisms A14013G (cytochrome B; CytB) and A9030G (cytochrome c oxidase subunit 1; COX1) with deltamethrin (DTM) resistance in *L. salmonis*. Salmon lice from Scottish aquaculture sites were classified as DM susceptible (S) or resistant (R) based on bioassays. Susceptible and resistant lice were subjected to allele specific PCR genotyping at SNPs A14013G and A9030G. CS: Classification success of A14013G or A9030G for DTM sensitivity.

Origin of *L. salmonis*	**DTM resistance**[†]	**n**	**Allele frequencies**		***P*-value**[‡] Allelic differentiation	**CS (%)** of 14013G or 3090G
			A	**G**		
Argyll 18	S	3	0.33	0.67	0.17	85
	R	51	0.12	0.88		
Argyll 19	S	7	0.43	0.57	0.05	75
	R	21[‡]	0.14	0.86		
Sutherland 19	S	10	0.70	0.30	0.002	73
	R	20	0.25	0.75		
Total	**S**	**20**	**0.55**	**0.45**	**<0.001**	**79**
	R	**92**	**0.15**	**0.85**		

[†]Susceptibility to deltamethrin was determined in single-dose bioassays, involving exposure (30 min) to 2 µg L^{-1} deltamethrin, followed by recovery in seawater (24 h) and subsequent rating as susceptible (affected) or resistant (unaffected).
[‡]21 out of 37 deltamethrin resistant individuals were subjected to allele specific PCR genotyping at SNPs A14013G and A9030G.

2.5 Discussion

The voltage gated sodium channel, Na_v, is considered the main target site for the toxicity of pyrethroids in arthropods, and resistance to insecticides of this class can be based on specific mutations of Na_v called *kdr* mutations. Three homologues of Na_v, called $LsNa_v1.1$-1.3, have been identified in *L. salmonis* in an earlier study, and a putative *kdr* mutation in $LsNa_v1.3$ (I936V; numbering according to *M. domestica* Na_v1) has been suggested as a potential genetic determinant of pyrethroid resistance in this species (Carmona-Antoñanzas et al., 2018). However, resistance of *L. salmonis* to the pyrethroid deltamethrin has previously been shown to be inherited maternally and to be associated with mitochondrial mutations, arguing against a primary role of Na_v in the resistance mechanism (Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015). The present study investigated whether $LsNa_v1.3$ I936V is involved in deltamethrin resistance in *L. salmonis*, using pharmacological and genetic approaches to differentiate its effects from those of mitochondrial mutations. Taken together, the results obtained do not provide evidence for major roles of $LsNa_v1.3$ I936V in deltamethrin resistance of *L. salmonis*.

Na_v comprises four highly conserved homologous domains (DI-DIV), each consisting of six transmembrane helices (S1-S6) connected by helical linkers (L) (Catterall, 1992). *Kdr* mutations are non-synonymous point mutations of Na_v that diminish its susceptibility to pyrethroids by altering the channel's gating kinetics and/or reducing its binding affinity for pyrethroids (Oliveira et al., 2013; Tan et al., 2005; Vais et al., 2003, 2000). To date, more than 50 *kdr* mutations/combinations of *kdr* mutations have been identified, some of which evolved independently in different arthropod species (Dong et al., 2014). Most *kdr* mutations map to two pyrethroid-binding sites within Na_v, predicted from homology models. One site involves L4-5 and S5 of DII and S6 of DIII (O'Reilly et al., 2006) and contains the I936V mutation investigated in this report, whereas the second site maps to L4-5 and S5 of DI and S6 of DII (Du et al., 2013) and harbours the first isolated *kdr* mutation L1041F (numbering according to *M. domestica* Na_v1) (Williamson et al., 1996). Deltamethrin is well studied regarding its interaction with Na_v in insects, where molecular docking studies predict it to bind to both pyrethroid-binding sites of the channel (Du et al., 2013; O'Reilly et al., 2006). Electrophysiological characterisation of mutant *D. melanogaster* Na_v expressed in *Xenopus* oocytes revealed that the mutation I936V reduces the channel's sensitivity to deltamethrin (Usherwood et al., 2007). Assuming similar effects of this mutation in the context of $LsNa_v1.3$, and further assuming that this *L. salmonis* Na_v homologue plays a role as a target-site for deltamethrin toxicity, *L. salmonis* expressing I936V $LsNa_v1.3$ would be expected to show a decreased deltamethrin susceptibility compared to parasites expressing the wild type channel.

To distinguish potential effects of I936V LsNa$_v$1.3 from those of the mitochondrial haplotype associated with deltamethrin resistance (Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015), parasites from a previously described crossing experiment were genotyped. In the experiment, families derived from crosses between females of the deltamethrin resistant strain IoA-02 and males of the drug-susceptible strain IoA-00 produced F1 and F2 generations in which all parasites were deltamethrin resistant (Carmona-Antoñanzas et al., 2017). While in families derived from crosses of the inverse orientation (IoA-00 female x IoA-02 male) most F2 parasites were deltamethrin susceptible, ~20% of F2 parasites were deltamethrin resistant in one of two families of this orientation, suggesting that nuclear genetic determinants of deltamethrin resistance had been transmitted by the IoA-02 male. However, genotyping of available F2 individuals from this family (n=69) in the current study revealed no difference in the I936V LsNa$_v$1.3 allele or genotype frequencies between deltamethrin resistant and drug susceptible F2 parasites. This finding suggests that the LsNa$_v$1.3 locus was not a genetic determinant of deltamethrin resistance in the cross. The reason for the lack of association of the I936V LsNa$_v$1.3 mutation with deltamethrin susceptibility in the cross is unknown. The residue Ile^{936} lies in the highly conserved S5 helix of DII, which forms part of the first proposed pyrethroid binding site of Na$_v$ (O'Reilly et al., 2006). Three conserved residues of arthropods Na$_v$, Cys^{933}, Ile^{936} and Phe^{1530}, show a divergent substitution at the homologous positions of the vertebrate Na$_v$ (Alanine (Ala, A), Valine (Val, V) and Ile, respectively), and have been suggested to contribute to the lower affinity of the vertebrate channel to pyrethroids (O'Reilly et al., 2006). Moreover, as stated above, effects of the I936V mutation on the deltamethrin susceptibility of fruit fly Na$_v$ have been confirmed by electrophysiological characterisation of recombinant channels expressed in *Xenopus* oocytes (Usherwood et al., 2007). Accordingly, it appears likely that I936V may also affect deltamethrin in the context of LsNa$_v$1.3. However, the cellular localisation and functional role of LsNa$_v$1.3 is unknown, and the channel may have no or only secondary relevance as a molecular target site for acute toxic effects of deltamethrin in adult and preadult *L. salmonis*. Deltamethrin toxicity in these parasite states could potentially be mainly mediated through other molecular targets, such as further Na$_v$ homologues LsNa$_v$1.1 and/or LsNa$_v$1.2, or hypothetical mitochondrial targets.

Further experiments were conducted with the non-ester pyrethroid etofenprox, whose chemical structure contains an ether bridge replacing the central ester group present in conventional pyrethroids. Etofenprox shares the m-phenoxybenzyl alcohol moiety with deltamethrin while possessing an acid moiety resembling that of fenvalerate (Nishimura et al., 1986). Ligand docking studies on a *M. domestica* Na$_v$ model revealed similar binding positions of fenvalerate, the base molecule for etofenprox, and deltamethrin (O'Reilly et al., 2006; Usherwood et al., 2007).

Several *kdr* mutations have been reported to confer cross-resistance to etofenprox. For example, in *M. domestica*, both L1041F and L1041F/M918T were linked to reduced sensitivity to etofenprox, fenvalerate, and deltamethrin (Beddie et al., 1996; Farnham et al., 1987; Pedersen, 1986; Soderlund, 2008). In the present study, the deltamethrin resistant *L. salmonis* strain IoA-02 did not differ in susceptibility to etofenprox when compared to a deltamethrin-susceptible reference strain. This finding supports the hypothesis that target-site mutations of *L. salmonis* Na_v homologues do not play a major role as determinants of deltamethrin resistance in *L. salmonis*.

This study further assessed deltamethrin susceptibility and its association with candidate genetic markers in five *L. salmonis* populations sampled at commercial aquaculture sites on the west coast of Scotland in 2017 to 2019. Deltamethrin EC_{50} values obtained (range: 1.6 - 8.0 µg L^{-1}) were significantly higher than values previously reported for the deltamethrin susceptible IoA-00 strain (0.28 µg L^{-1}, 95% confidence limits: 0.23 - 0.36 µg L^{-1}) (Carmona-Antoñanzas et al., 2017), suggesting that deltamethrin resistance is widespread in *L. salmonis* populations of the Scottish west coasts, confirming the results of earlier studies (Carmona-Antoñanzas et al., 2017; Fjørtoft et al., 2020). Parasites (n=102) for which individual deltamethrin susceptibility phenotypes were available (resistance criterion: no behavioural signs of toxicity after 30 min of exposure to 2.0 µg L^{-1} deltamethrin and 24 h of recovery) were genotyped for the $LsNa_v1.3$ I936V mutation and two mitochondrial SNPs allowing detection of the pyrethroid resistance-associated mitochondrial haplotype. Deltamethrin resistant and susceptible *L. salmonis* obtained from field sites did not differ in allele and genotype frequencies for the $LsNa_v1.3$ I936V locus, which is in line with the above results obtained from genotyping of resistant and susceptible F2 parasites but contrasts previously published findings (Carmona-Antoñanzas et al., 2018).

In contrast to $LsNa_v1.3$ I936V, both mitochondrial markers A14013G (CytB) and A9030G (COX1) significantly differentiated deltamethrin resistant and susceptible parasites for all aquaculture production sites, except for one population in which very few susceptible parasites had been obtained, lowering testing power. These results support the hypothesis that deltamethrin resistance in Scottish field populations of *L. salmonis* involved mitochondrial genetic determinants and can be reliably monitored by mitochondrial SNP markers proposed in earlier studies (Carmona-Antoñanzas et al., 2017). The mtDNA SNPs A14013G and A9030G used in this study are markers of a previously defined deltamethrin resistance-associated mtDNA haplotype involving further 26 SNPs (Carmona-Antoñanzas et al., 2017). It has been suggested that the resistance-associated mitochondrial haplotype first emerged around 2009, when it was detectable in parasites from aquaculture sites in Ireland, the Shetland isles and Norway (Fjørtoft et al., 2020). However, moderate levels of deltamethrin resistance had already been

reported in the early 2000s, with EC_{50} values of up to 1.03 µg L^{-1} determined for *L. salmonis* populations sampled in 2001 to 2003 at Norwegian and Irish sites (Sevatdal et al., 2005a).
The mechanism underlying the association of mitochondrial genetic markers with deltamethrin resistance is still unresolved. It has been suggested that deltamethrin may disrupt mitochondrial function, a hypothesis supported by ATP-depleting and apoptosis-inducing effects of the drug in *L. salmonis* (Bakke et al., 2018; Carmona-Antoñanzas et al., 2017), but the molecular target of the proposed mitochondrial effects remains to be identified. The two mtDNA SNPs investigated in this study are corresponding to synonymous mutations. To date, an impact on deltamethrin resistance cannot be ruled out completely. Synonymous mutations may play a role in altering gene functions, including gene expression (Wang et al., 2005), the formation of secondary structures of proteins (Gupta et al., 2000), protein folding, and substrate/protein interaction (Kimchi-Sarfaty et al., 2007). However, most likely, mtDNA SNPs A14013G and A9030G are only non-causally linked to deltamethrin resistance due to the lack of recombination in mtDNA.

Although results from this study support the view that mitochondrial mutations play a predominant role among genetic factors causing deltamethrin resistance in *L. salmonis*, they also provide evidence for the involvement of further nuclear determinants. Of the parasites analysed, ~20% of those from the crossing experiment and ~15% of those originating from field sites were resistant but lacked the deltamethrin resistance-associated mtDNA haplotype, suggesting the contribution of nuclear genes to deltamethrin resistance. In terrestrial arthropods, deltamethrin resistance can be conferred by target-site mutations of Na_v (Knipple et al., 1994; Williamson et al., 1993) and enhanced enzymatic detoxification (Ranson et al., 2002). In a previous study, three Na_v homologues were identified in the *L. salmonis* genome, and SNPs in conserved regions of these channels determined by cDNA sequencing of deltamethrin resistant and susceptible parasites (Carmona-Antoñanzas et al., 2018). The present study provides an in-depth investigation of $LsNa_v1.3$ I936V, a SNPs identified in the previous study, but results argue against relevance of this mutation for deltamethrin resistance in the studied parasites. In terrestrial arthropods, pyrethroid resistance based on metabolic detoxification usually involves the enhanced expression of biotransformation enzymes, such as CYPs, esterases, or GSTs (Panini et al., 2016). For example, pyrethroid resistance in *Anopheles funestus* did not involve Na_v mutations but was linked to overexpression of CYPs and GSTs (Atoyebi et al., 2020; Irving and Wondji, 2017; Morgan et al., 2010). Similarly, metabolic resistance has been implicated in pyrethroid resistance in isolates of *Aedes albopictus* (Ishak et al., 2016)*, A. arabiensis* (Witzig et al., 2013), and *M. domestica* (Nicholson and Sawicki, 1982). Several studies have investigated the transcriptional responses of caligid sea lice to pyrethroid exposure, demonstrating effects on transcript expression of CYPs (Humble et al., 2019; Poley et al., 2016), serine proteases (Poley et

al., 2016; Valenzuela-Miranda and Gallardo-Escárate, 2016), and antioxidant enzymes (Chavez-Mardones and Gallardo-Escárate, 2014; Poley et al., 2016). The CYP gene superfamily has been characterised in *L. salmonis,* but no constitutive upregulation of transcript expression was found in comparative studies of deltamethrin resistant and susceptible parasites (Humble et al., 2019). Insect populations in which pyrethroid resistance is based on CYP overexpression often show cross-resistance to etofenprox (Arouri et al., 2015; Tan and McCaffery, 2007), whose chemical structure impedes its metabolic detoxification by esterases and GSTs (Hemingway, 1995; Karunaratne et al., 2007). However, the lack of evidence for a constitutive upregulation of CYP genes in the IoA-02 strain is in accordance with a lack of cross-resistance of the deltamethrin resistant *L. salmonis* strain IoA-02 to etofenprox.

Further gene families potentially involved in metabolic insecticide resistance remain to be characterised in *L. salmonis*.

Conclusion

In the present study, the mutation I936V of *L. salmonis* sodium channel LsNa$_v$1.3 showed no association with deltamethrin resistance, as defined based on the results of acute toxicity tests carried out with adult/preadult parasites. However, protective roles of the mutation cannot be excluded with other exposure scenarios, or for other life stages. Results of the study further confirm previous reports of an association of deltamethrin resistance with mtDNA mutations in *L. salmonis*.

Chapter 3

Key role of mitochondrial mutation Leu107Ser (COX1) in deltamethrin resistance in salmon lice (*Lepeophtheirus salmonis*)

3.1 Abstract

Deltamethrin is used to treat Atlantic salmon (*Salmo salar*) against salmon lice (*Lepeophtheirus salmonis*) infestations. However, development of deltamethrin resistance has been reported from North Atlantic *L. salmonis* populations, in which resistance is associated with mtDNA mutations. This study investigated the relationship between deltamethrin resistance and mtDNA SNPs. A total of 188 *L. salmonis* collected from Scottish aquaculture sites were assessed using deltamethrin bioassays and genotyped at 18 SNP loci. Genotyping further included archived parasites of known deltamethrin susceptibility status. The results identified eleven mtDNA haplotypes, three of which were associated with deltamethrin resistance. Phylogenetic analyses of haplotypes suggested multiple origins of deltamethrin resistance. *L. salmonis* laboratory strains IoA-00 and IoA-10 showed similarly high levels (~100-fold) of deltamethrin resistance in bioassays. Both strains differed strongly in mtDNA haplotype but shared the missense mutation Leu107Ser in the mitochondrial gene COX1, which was detected in all further deltamethrin resistant *L. salmonis* isolates assessed. In crossing experiments with a deltamethrin susceptible strains, maternal inheritance of deltamethrin resistance is apparent with both IoA-10 (this study) and IoA-02 (earlier reports). We conclude that Leu107Ser (COX1) is a main genetic determinant of deltamethrin resistance in *L. salmonis.*

3.2 Introduction

Caligid sea lice (Copepoda: Crustacea) are ectoparasite of marine fish, which feed on the mucus, skin and blood of their hosts (Boxaspen, 2006). In the most severe cases, infestations can lead to skin lesions, secondary infections, osmoregulatory imbalances, induction of endocrine stress responses, reduced appetite and growth, immunosuppression and, if untreated, potentially death (Grimnes and Jakobsen, 1996; Wootten et al., 1982). Sea lice infections are a major health management problem in the commercial mariculture of Atlantic salmon (*Salmo salar*), with the salmon louse *Lepeophtheirus salmonis* causing most infestation in the North Atlantic. In 2018, the estimated global costs of sea lice infections to the salmon industry were approximately US $873 million/£700 million (Brooker et al., 2018b), comprising mainly treatment costs and to a lesser extent loss in production.

Sea lice infections of farmed salmon are controlled by integrated pest management strategies combining farm management measures, such as fallowing and the use of single year classes, with a diverse array of non-medicinal control approaches and a limited range of licensed

veterinary drugs (Burridge et al., 2010; Torrissen et al., 2013). Non-medicinal sea lice control approaches include alternative cage designs reducing infection pressure (Stien et al., 2016), different systems of salmon delousing using physical means such as the application of water jets or brief immersion in warm water (Overton et al., 2019), and the co-culture of Atlantic salmon with different species of cleaner fish that remove sea lice from infected salmon (Brooker et al., 2018a). Veterinary drugs available for salmon delousing include the macrocyclic lactone emamectin benzoate and different benzoylureas, which are applied as feed additives, and the organophosphate azamethiphos, the disinfectant hydrogen peroxide, and the pyrethroid deltamethrin, which are supplied as bath treatments (Burridge et al., 2010).

The use of a limited range of salmon delousing agents over more than two decades has led to the evolution of drug resistance in *L. salmonis* populations of the North Atlantic, with most current veterinary treatments being affected by losses of efficacy (Aaen et al., 2015; Jensen et al., 2020; Torrissen et al., 2013). The pyrethroid deltamethrin has been in use as bath treatment against sea lice infections of Atlantic salmon since 1998. While moderate losses of susceptibility were reported in the early 2000s (Sevatdal and Horsberg, 2003), higher levels of resistance have been described more recently (Carmona-Antoñanzas et al., 2017). The toxicity of pyrethroids is based on their blocking of arthropod voltage-gated sodium channels (Na_v), which have essential roles in neurotransmission (Davies et al., 2007). Pyrethroid resistance in insects can be based on missense mutations of Na_v that decrease the channel's affinity to the pesticides (Knipple et al., 1994; Williamson et al., 1993), or result from the constitutive up-regulation of key enzymes of pesticide detoxification, such as the carboxylesterases (CaEs), cytochrome P450s (CYPs), or glutathione-S-transferases (Ranson et al., 2002).

In *L. salmonis,* the molecular mechanism of pyrethroid resistance remains to be resolved. Deltamethrin resistance in *L. salmonis* shows a predominantly maternal mode of inheritance, and is associated with mitochondrial mutations (Bakke et al., 2018; Carmona-Antoñanzas et al., 2017). These findings suggest a novel, still unresolved resistance mechanism and imply still unidentified mitochondrial targets for deltamethrin toxicity in susceptible lice. In support of this hypothesis, deltamethrin has been shown to disrupt mitochondrial ATP production (Carmona-Antoñanzas et al., 2017) and induce apoptosis in skeletal muscle, which have a high content of mitochondria (Bakke et al., 2018). Results from crossing experiments suggested additional minor roles of unidentified nuclear genetic factors in deltamethrin susceptibility (Carmona-Antoñanzas et al., 2017). In *L. salmonis,* three genes encoding Na_v channels have been identified (Carmona-Antoñanzas et al., 2018), one of which harbours a SNP that is homologous to a mutation of insect Na_v decreasing pyrethroid affinity (Usherwood et al., 2007). However, in *L. salmonis* field populations the Na_v SNP showed no association with deltamethrin resistance

(Tschesche et al., 2020). Similarly, the genomic wide characterization and transcript expression studies of *L. salmonis* CYP and CaE genes failed to provide evidence for an upregulation of CYPs or CaEs in deltamethrin resistant parasites (Humble et al., 2019; Tschesche et al., 2021).

In pest or parasite populations, mutations causing pesticide resistance can form *de novo* or be introduced through migration (Ffrench-Constant et al., 2004). Studies determining whether resistance-associated mutations have single or multiple origins can provide insights into the relative importance of mutation as compared to migration in the formation of resistance. Genetic variation between *L. salmonis* populations is weak throughout the North Atlantic, suggesting the species forms a single panmictic population in this geographic area (Besnier et al., 2014; Glover et al., 2011). The low degree of genetic differentiation in Atlantic *L. salmonis* most likely results from the migratory nature of its main host, Atlantic salmon, in which populations reproducing in different freshwater systems share common oceanic feeding grounds during the marine phase of the salmon life cycle (Jacobsen et al., 2012), providing an opportunity for *L. salmonis* cross-infections. Further contributing to the genetic exchange between *L. salmonis* populations at a more local scale is the parasite's planktonic dispersal at nauplii and copepodid larval stages, which has been estimated to allow for transmission of up to 45 km (Johnsen et al., 2016).

In accordance with high rates of gene flow between Atlantic *L. salmonis*, the analysis of haplotypes associated with emamectin benzoate hyposensitivity suggested that resistance against this drug has arisen once through *de novo* mutation followed by a rapid spread throughout the North Atlantic (Besnier et al., 2014). In contrast, the analysis of SNPs closely linked to a mutation in the *L. salmonis ace1a* gene, previously shown to confer azamethiphos resistance (Kaur et al., 2015a), revealed multiple and divergent haplotypes associated with resistance (Kaur et al., 2017). This suggests that the *ace1a* mutation causing resistance existed prior to the introduction of organophosphate salmon delousing agents, the use of which caused the parallel selection of different haplotypes containing the mutation. In a study of deltamethrin resistance in *L. salmonis,* four independent highly resistant strain isolates originating from geographically distant Scottish farm sites showed virtually identical mitochondrial genome sequences, contrasting highly polymorphic mitochondrial genome sequences among deltamethrin susceptible samples (Carmona-Antoñanzas et al., 2017). While these findings suggest the clonal expansion of deltamethrin-resistance-associated mitochondrial haplotypes as a result of drug selection, the study may have missed rare haplotypes present due to the limited number of individuals analysed.

The present study had the objective of characterising mitochondrial DNA (mtDNA) haplotypes in field populations of *L. salmonis* and assessing their relationship to deltamethrin resistance. *L. salmonis* were obtained from a range of Scottish farm sites and subjected to bioassays to determine their deltamethrin susceptibility status. Parasites were then genotyped at selected mitochondrial SNP loci to identify mitochondrial haplotypes and assess haplotype association with deltamethrin resistance. Mitochondrial haplotypes were compared to infer whether deltamethrin resistance in salmon lice evolved from single or multiple origins. One resistance-associated haplotype, which differed maximally from a previously studied haplotype, was selected for further investigations. A laboratory *L. salmonis* strain carrying the haplotype of interest was established and its mitochondrial genome sequenced. The mtDNA of that strain was compared to the mtDNA of previously analysed deltamethrin resistant and susceptible *L. salmonis* strains to derive hypotheses about the potential involvement of mitochondrial SNPs in the resistance mechanisms. The deltamethrin susceptibility of the newly established strain was characterised using drug bioassays and effects of sublethal deltamethrin concentrations on total ATP levels were assessed. The strain was further subjected to crossing experiments to assess the mode of inheritance of deltamethrin resistance.

3.3 Materials and methods

3.3.1 Ethics statement

All research projects involving the University of Stirling are subject to a thorough Ethical Review Process prior to any work being approved. The present research was assessed by the University of Stirling AWERB and passed the ethical review process. Laboratory infections of Atlantic salmon with *L. salmonis* were performed under a valid UK Home Office license and at low parasite densities unlikely to compromise fish welfare.

3.3.2 *Lepeophtheirus salmonis*

L. salmonis strains were maintained under standardised conditions at the Marine Environmental Research Laboratory of the University of Stirling (Machrihanish, UK), as described in detail elsewhere (Heumann et al., 2012). Strain IoA-00 was derived from an isolate collected in the Firth of Clyde system in 2003 and is susceptible to DTM, emamectin benzoate, and azamethiphos (Carmona-Antoñanzas et al., 2017, 2016; Humble et al., 2019). Strain IoA-01, which originates from Sutherland and was taken into culture in 2008, is resistant to emamectin benzoate but susceptible to DTM (Carmona-Antoñanzas et al., 2017, 2016; Humble et al., 2019). Strain IoA-02 was established in 2011 from an isolate collected in the Shetland islands and is resistant to emamectin benzoate, DTM, and azamethiphos (Carmona-Antoñanzas et al., 2017, 2016; Humble

et al., 2019). Strains IoA-03 (Sutherland, 2014), NA01-O and NA01-P (Argyll and Bute, 2012) are further DTM resistant (Carmona-Antoñanzas et al., 2017).

Strain IoA-10 was established during this study. Gravid female *L. salmonis* (N=30) were obtained from an aquaculture production site located on the West coast of Scotland (Inverness-shire, 2019). Lice were put into polyethylene bags containing cool (~10°C) oxygen-saturated seawater, which were placed in insulated boxes equipped with cold packs and immediately shipped to MERL. Paired egg strings were removed from dams and incubated at ambient temperature in separate containers linked to airlines, to produce batches of copepodids derived from the same mother. The corresponding dams were preserved in absolute ethanol, and subjected to DNA extraction and genotyping assays to establish mtDNA haplotypes (see section 3.3.4). Copepodids derived from one dam showing the haplotype of interest were selected and used to infect naïve host fish to establish strain IoA-10 after the mtDNA haplotype of the larval batch had been ascertain by repeating genotyping assays with a subset of copepodids.

This study further included preadult-II and adult *L. salmonis* that were collected from two Scottish aquaculture sites in Argyll and Bute (farm site 1: 2018, N=206; farm site 2: 2018, N=54). Lice were shipped to the University of Stirling, as described above, transferred to a temperature-controlled incubator set to 12°C, and linked to airlines. Lice were used to set up DTM bioassays (see section 3.3.3) the following day, at the end of which parasites were removed and conserved in absolute ethanol for later genotyping (see section 3.3.4).

An industry partner further provided ethanol-conserved preadult-II and adult salmon lice for molecular analyses, which were collected from farm sites in Argyll and Bute (farm site 3: 2019, N=176) and Sutherland (farm site 4: 2019, N=148) and phenotyped by DTM bioassays.

To obtain *L. salmonis* for copepodid bioassays (see section 3.3.3), gravid lice with paired egg strings were collected from Argyll and Bute (farm sites 5: 2019, N=1) and Inverness-shire (farm site 6: 2019, N=1; farm site and 7: 2019, N=1), followed by shipment to the University of Stirling and transfer of lice to a temperature-controlled incubator, as described above. To obtain batches of copepodids derived from the same dam, paired egg strings were removed from females and incubated in beakers containing seawater and linked to airlines in a temperature-controlled chamber set to 12°C, allowing hatching and progression through larval stages.

Further parasites studied included *L. salmonis* collected from wild host fish caught in a salmon river located on the East coast of Scotland (N=18) (Carmona-Antoñanzas et al., 2017), which were stored in absolute ethanol at -20°C pending molecular analyses.

3.3.3 *Lepeophtheirus salmonis* bioassays

Bioassays were performed to assess the susceptibility of both preadult-II/adult salmon lice and copepodid larvae lice to deltamethrin (Pestanal® analytical standard grade, Sigma-Aldrich, Gillingham, UK), which was dissolved in acetone at a final solvent concentration of 0.05% (v/v) in all tests. All bioassay incubations took place in a temperature-controlled chamber set to 12°C.

Bioassays with preadult/adult salmon lice performed within this study had either a standard or a single-dose design (Helgesen and Horsberg, 2013; Sevatdal and Horsberg, 2003), and have been described in detail elsewhere (Tschesche et al., 2020). In brief, *L. salmonis* adult males and preadult II/adult females were randomly allocated to 300 mL crystallising dishes containing 100 mL of filtered (55 µm) seawater, with each dish receiving five females and five males. Standard bioassays included a geometrical series of at least six deltamethrin concentrations in the range of 0.125 and 32 µg L^{-1} and a solvent control (Table S3.1) in duplicates. Single-dose bioassays were conducted in an analogous fashion, except that deltamethrin was provided at one diagnostic concentration (2 µg L^{-1}). Preadult/adult salmon lice were exposed to deltamethrin for 30 min and then allowed to recover in clean seawater for 24 h prior to behavioural responses being examined and rated as “live”, “weak”, “moribund”, or “dead”. Rating criteria have been described in detail elsewhere (Carmona-Antoñanzas et al., 2016).

L. salmonis copepodids for copepodid bioassays were generated by incubation of egg strings (see section 3.3.2). A 1 mL pipette was used to randomly allocate 1 mL of filtered seawater with approximately ten copepodids to embryo dishes with glass lids containing 1 mL of filtered seawater. Chemical exposures were initiated by adding 1 µL of a 2000x final concentration solution of deltamethrin to the embryo dishes containing 2 mL seawater and parasites. Copepodids were exposed to nine concentrations of deltamethrin (0.10, 0.22, 0.46, 1.00, 2.15, 4.64, 10.00, 21.54, and 46.42 µg L^{-1}) and one solvent control, in duplicates. Deltamethrin exposure was for 24 h, directly followed by examination and rating of larvae. Copepodids were rated “live” when attracted by light and swimming normally, “weak” when swimming irregularly (animals swim in a straight line within 2 min after stimulation using light and a fine brush), “moribund” when incapable of swimming away after stimulation by light and a fine brush (animals may twitch appendages), and “dead” when showing no movements in extremities, gut, or other organs as apparent from examination under a microscope.

Copepodid, preadult, and adult salmon lice rated as “live” or “weak” were considered unaffected, while “moribund” and “dead” parasites were considered affected. In bioassays involving exposure to a series of drug concentrations the susceptibility of the tested population was characterised by probit analysis, while in single-dose bioassays involving exposure to 2 µg L^{-1}

deltamethrin parasites were classified as deltamethrin resistant if rated unaffected and susceptible if rated affected. After completion of bioassays, lice were stored in absolute ethanol at -20°C pending DNA extraction and genetic analyses.

3.3.4 Genotyping of mitochondrial single nucleotide polymorphism (SNP) alleles

PCR based genotyping assays employing universal FRET probes (KASP® 4.0, LGC Genomics, Teddington, UK) were designed to detect 18 mtDNA SNPs, including the four non-synonymous SNPs G8134A, T5889C, T8600C, and G3338A (Numbering according to NCBI accession number LT630766.1), that have been associated with deltamethrin resistance in *L. salmonis* in a previous study (Carmona-Antoñanzas et al., 2017). Genomic DNA was extracted from individual *L. salmonis* specimens using a high throughput protocol (Montero-Pau et al., 2008), with details having been reported elsewhere (Tschesche et al., 2020). Each SNP assay involved one common primer and two allele specific primers (Table S3.2), with the method being described in detail elsewhere (Tschesche et al., 2020).

3.3.5 Mitochondrial haplotype network

A haplotype network was interfered from mitochondrial haplotypes of *L. salmonis*. The network was constructed using the medium-joining method (Bandelt et al., 1999) implemented in the software PopArt v1.7 (Leigh and Bryant, 2015). Haplotypes were defined based on the combined occurrence of 18 SNPs that have been associated with deltamethrin resistance in *L. salmonis* in a previous study (Carmona-Antoñanzas et al., 2017).

3.3.6 Mitochondrial genome (mtDNA) amplification and sequencing

The mitochondrial genome was amplified and sequenced from two salmon lice from the laboratory-maintained strain IoA-10. The *L. salmonis* mitochondrial genome was amplified in six overlapping PCRs products using specific oligonucleotide primers that have been designed with Primer3 v4.1.0 (https://primer3.ut.ee/) (Table S3.3). PCR reactions were performed using 1 µl of 50 ng μL^{-1} template DNA, 2.5 µL (10 pmol) of the forward and the reverse primer, 25 µL Q5® High-Fidelity 2x Master Mix (New England BioLabs Ltd, Hitchin, UK), and 19 µL nuclease-free water. PCR conditions for each product are listed in Table S3.4. All PCR products were purified (NucleoSpin™ Gel and PCR Clean-up Kit, Macherey-Nagel, Düren, Germany) and sequenced (Table S3.5) by Eurofins Genomics (Ebersberg, Germany). Prior to sequencing, PCR product 6 (Table S3.3) had to be subcloned using a pGEM-T Easy Vector system and chemo-competent *Escherichia coli* JM-109 (Promega, WI, USA), and plasmids were isolated using a NucleoSpin™ Plasmid EasyPure kit (Macherey-Nagel, Düren, Germany). MtDNA sequences obtained for the same individual were manually assessed and trimmed and aligned.

3.3.7 Identification of deltamethrin resistance-associated mtDNA SNP

MtDNA sequences from IoA-10 lice, as well as archived sequences from individuals of strains IoA-00 (N = 2), IoA-02 (N = 2), IoA-01 (N = 4), NA01-P (N = 2) and NA01-O (N = 3), wild hosts (N = 2), and F2 lice from IoA-00 and IoA-02 crosses (N = 12) (Carmona-Antoñanzas et al., 2017), were aligned to the Scottish *L. salmonis* mitochondrial reference genome (NCBI accession number LT630766.1) using the R/Bioconductor package Rbowtie2 v 4.0.3 (Wei et al., 2018). Sequence variations were identified using the *HaplotypeCaller* function in GATK v3.5 (McKenna et al., 2010). IoA-10 specific SNPs, as well as SNPs common to all deltamethrin resistant individuals and lacking in all susceptible parasites were then identified.

3.3.8 *Lepeophtheirus salmonis* crosses

The laboratory strains IoA-00, IoA-02, and IoA-10 were crossed to produce five families spanning two generations termed parental (P0) and first filial generation (F1). IoA-00, IoA-02, and IoA-10 copepodids were maintained on Atlantic salmon smolts in separate tanks to allow development to the adult male and preadult-II female stage. Then, half of the fish carrying IoA-00 lice and half of the fish carrying IoA-10 lice were humanely killed to harvest lice in order to set up two batch crosses. In the first batch cross the P0 generation consisted of IoA-00 adult males and IoA-10 preadult-II females, whereas the P0 generation in the second batch cross had the inverse gender-strain orientation. To set up each batch cross, ten Atlantic salmon smolts were anaesthetised using 2-phenoxyethanol (100 mg L^{-1}; 99%; Sigma-Aldrich, Gillingham, UK) and received two to three male and female parasites, respectively. The remaining fish carrying IoA-00 adult males/preadult-II females and IoA-10 adult males/preadult-II females, as well as all fish carrying IoA-02 adult males/preadult-II females were maintained further, allowing lice to reproduce separately. P0 dams of the five families were maintained to produce egg strings, which were then removed and incubated to allow hatching and larval development (Heumann et al., 2014). The resulting F1 copepodid larvae were used to inoculate tanks containing naïve Atlantic salmon. Infections were maintained until F1 parasites reached the adult male and preadult-II female stages. Subsequently, F1 parasites were subjected to bioassays to determine their deltamethrin susceptibility phenotype.

3.3.9 Effects of deltamethrin on *L. salmonis* ATP levels

Whole body ATP levels were assessed in *L. salmonis* from strains IoA-00, IoA-02, and IoA-10 following exposure to sublethal concentrations of deltamethrin and fenpyroximate (Pestanal® analytical standard grade, Sigma-Aldrich, Gillingham, UK). The acaricide fenpyroximate served as a positive control, as it is known to act by interference with the mitochondrial complex I (Lümmen, 1998).

Drug exposures in ATP experiments followed a similar methodology to that used for bioassays. Adult male salmon lice from strains IoA-00, IoA-02, and IoA-10 were randomly allocated to crystallising dishes containing 100 mL of filtered seawater, with each dish receiving five adult males. Deltamethrin and fenpyroximate were solubilised in acetone (0.05% v/v in all tests). Parasites were exposed to a solvent control and sublethal concentrations of the tested compounds (deltamethrin: 2 µg L^{-1}; fenpyroximate: 100 µg L^{-1}) in quadruplicate test dishes. Adult males were exposed to deltamethrin for 30 min and then allowed to recover in clean seawater for 300 min. Exposure to fenpyroximate was for 300 min, without seawater recovery. After drug exposure/seawater recovery, the behavioural response of each animal was rated according to categories given above, and animals deemed alive were added to separate plastic tube containing 1 mL of Tris–EDTA-saturated phenol (10 mM Tris HCl and 1 mM EDTA; Sigma-Aldrich, Gillingham, UK; Thistle Scientific Ltd, Glasgow, UK). Samples were incubated at room temperature for 10 min and subsequently stored at -70°C. After completion of sampling, frozen samples were submerged in dry ice and transported to the University of Stirling pending ATP analyses. Whole-body ATP levels were measured using a commercially available luciferin-luciferase bioluminescence assay kit (A-22066, Molecular Probes, Thermo Fisher Scientific, Bishop's Stortford, UK), with details having been reported elsewhere (Carmona-Antoñanzas et al., 2017).

3.3.10 Alignment of ND1, ND5, COX1, and COX3 among crustacean species

Amino acid sequences of ND1 and ND5, and COX1 and COX3 from *L. salmonis* and other crustaceans (NCBI accession numbers: *Caligus clemensis* HQ157566.1, *Caligus rogercresseyi* HQ157565.1, *Tigriopus caligornicus* DQ913891.2, *Paracyclopina nana* EU877959.1, *Calanus hyperboreus* NC_019627.1, *Eucalanus bungii* AB091772.1, *Squilla mantis* NC_006081.1, *Thalassomya japonica* NC_008974.1, *Homarus americanus* NC_015607.1, *Vargulla hilgendorfii* NC_005306.1, *Daphnia pulex* NC_000844.1, *Artemia franciscana* NC_001620.1, *Triops cancriformis* NC_004465.1) were aligned using default parameters in the online software Clustal Omega version 2.1 (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers and Higgins, 2018).

3.3.11 Data analyses and statistical tests

The dose-response relationship for deltamethrin tested in *L. salmonis* bioassays was assessed by probit analysis using the statistical program R version 4.0.2 (package *drc*), assuming a log-normal distribution of drug susceptibility. Based on the fitted models, EC_{50} and 95% confidence limits were derived and effects of sex and origin/strain on drug susceptibility assessed.

Whole body ATP levels were expressed as means ± standard error (n = 15). All statistical analyses were performed in R version 4.0.2 (packages car, rcompanion, PMCMR). Data were tested for normality and homogeneity of variance using Shapiro-Wilk's and Levene's tests, respectively. As a number of ATP data sets violated these assumptions, the Kruskal-Wallis test was used to assess the effect of drug treatments on ATP levels. The significance level was set at $P < 0.05$. The experiment-wise type I error was controlled by sequential Bonferroni correction (Rice, 1989). In those cases where Kruskal-Wallis test results indicated significant differences between treatments, Dunn's test was employed for *post-hoc* comparisons to the control group.

3.4 Results

3.4.1 Phenotyping *L. salmonis* from aquaculture sites, genotyping at four SNP loci

In the years 2018 and 2019, *L. salmonis* were collected at four Scottish aquaculture sites, subjected to single-dose DTM bioassays to classify parasites as DTM resistant or DTM susceptible, and genotyped at four non-synonymous mtDNA SNP loci previously shown to be associated with DTM susceptibility (Carmona-Antoñanzas et al., 2017). In addition to field collected lice, genotyping included parasites of previously characterised laboratory-maintained strains IoA-00 (DTM-susceptible) and IoA-02 (DTM resistant). The tested SNPs comprised mtDNA mutations G8134A, T5889C, T8600C, and G3338A, which are located in ND1, ND5, COX1, and COX3, respectively. Based on the combined occurrence of these SNPs, four haplotypes were defined (Table 3.1). Genotyping results are summarised in Table 3.2. As reported previously, lice of the deltamethrin susceptible strain IoA-00 possess haplotype 1, defined by the wild type alleles T8600, G8134, T5889, and G3338, whereas parasites from the deltamethrin resistant strain IoA-02 show haplotype 2, comprising mutant alleles 3338A, 5889C, 8134A, and 8600C (Carmona-Antoñanzas et al., 2017). Genotyping of salmon lice from field populations revealed, in addition to haplotypes 1 and 2, two new haplotypes 3 and 4. Most animals with haplotypes 2, 3 and 4 were classified as deltamethrin resistant (93%, 87% and 100%, respectively). In contrast, 57% of individuals with haplotype 1 were rated deltamethrin susceptible, while 43% were classified resistant (Table 3.2).

Table 3.1. Haplotype definitions. Haplotypes 1, 2, 3, and 4 were defined based on the combined occurrence of the four non-synonymous single nucleotide polymorphisms G3338A, T5889C, G8134A, and T8600C, which have been linked to resistance in a previous study (Carmona-Antoñanzas et al., 2017).

Position†	Alleles	Haplotypes 1	Haplotypes 2	Haplotypes 3	Haplotypes 4
3338	G/A	G	A	G	G
5889	T/C	T	C	T	C
8134	G/A	G	A	G	A
8600	T/C	T	C	C	C

†Numbering according to the Scottish *L. salmonis* mitochondrial reference genome (NCBI Accession number LT630766.1).

Table 3.2 Association of mitochondrial haplotypes with deltamethrin resistance. *L. salmonis* of laboratory strains IoA-00 and IoA-02 and parasites obtained from Scottish aquaculture production sites were subjected to deltamethrin bioassays to establish susceptibility status, followed by genotyping at four SNP loci to establish haplotypes (see table 3.1 for details).

Origin of *L. salmonis*	Deltamethrin resistance†	N	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4
IoA-00	Susceptible	22	22	-	-	-
IoA-02	Resistant	24	-	24	-	-
Farm site 1	Susceptible	3	1	2	-	-
Argyll 2018	Resistant	103	3	76	16	8
Farm site 2	Susceptible	3	1	2	-	-
Argyll 2018	Resistant	51	2	39	4	6
Farm site 3	Susceptible	7	-	4	3	-
Argyll 2019	Resistant	21‡	-	16	3	2
Farm site 4	Susceptible	10	6	3	1	-
Sutherland 19	Resistant	20	1	15	4	-
Total		**218**	**N = 14**	**N = 157**	**N = 31**	**N = 16**
(Farm sites)	**Susceptible**	**23**	**57.1%**	**7%**	**12.9%**	**0%**
	Resistant	**195**	**42.9%**	**93%**	**87.1%**	**100%**

†Susceptibility to deltamethrin was determined in single-dose bioassays, involving exposure (30 min) to 2 µg L^{-1} deltamethrin, followed by recovery in seawater (24 h) and subsequent rating as susceptible (affected) or resistant (unaffected).
‡21 out of 37 deltamethrin resistant individuals were subjected to allele specific PCR genotyping.

3.4.2 Relationship of mtDNA haplotypes to deltamethrin resistance of copepodids

The relationship between mtDNA haplotypes and deltamethrin resistance was further investigated in 24 h behavioural bioassays with copepodid larvae derived from gravid females collected at field sites and from laboratory strains. The deltamethrin EC_{50} in haplotype 1 copepodids (laboratory strain IoA-00) was determined as 0.14 µg L^{-1}. For copepodid larvae possessing haplotype 2, estimates of the deltamethrin EC_{50} value were 4.81 µg L^{-1} (laboratory strain IoA-02) and 3.98 µg L^{-1} (field derived larvae), while copepodids having haplotype 3 showed an EC_{50} of 6.90 µg L^{-1}, field derived larvae (Table 3.3).

Table 3.3 Deltamethrin susceptibility and mitochondrial haplotype of *L. salmonis* copepodid larvae. Each bioassay was performed with the copepodid descendants of one female salmon louse. *L. salmonis* originated from the laboratory strains IoA-00 and IoA-02, or Scottish aquaculture production sites. Bioassays involved exposure (24 h) to deltamethrin and rating of copepodids as normal or affected. Haplotypes were established based on the genotyping of 10 individuals per bioassay.

Origin of *L. salmonis*	N bioassay	Haplotype†	EC_{50} (95% CI)‡ [µg L^{-1}]
IoA-00	6	1	0.14 (0.13-0.16)
IoA-02	3	2	4.81 (3.99-5.63)
Farm site 5, 6	2	2	3.98 (2.93-5.04)
Farm sites 5, 7	2	3	6.90 (4.15-9.65)

†For definition of haplotypes, see table 3.1.
‡Raw data used to derive EC_{50} values are provided in Table S3.10.

3.4.3 Further characterisation of mitochondrial haplotypes

To further characterise mtDNA diversity in *L. salmonis* populations of Scottish aquaculture sites, a subset of the above samples from field sites and strains IoA-00 and IoA-02 was further genotyped at 14 mtDNA SNP loci, corresponding to synonymous mutations previously found to be associated with deltamethrin resistance (Carmona-Antoñanzas et al., 2017) (Table S3.6 and S3.7). Genotyping further included *L. salmonis* collected from wild hosts in 2010 (N=18), as well as conserved specimens of the deltamethrin resistant strain NA01-O (N=20) (Carmona-Antoñanzas et al., 2017). Based on the combined occurrence of the total 18 SNPs considered, the above haplotype definition was refined. Haplotypes 2, 3 and 4 were confirmed by the wider range of SNPs, while haplotype 1 defined on the basis of the initially tested four non-synonymous SNPs was subdivided based on the wider panel of SNPs, differentiating eight new haplotypes named 1A to 1H (Table S3.6). Resistance-associated haplotype 4 was found to be present in specimens removed from wild fish in 2010 and both resistance-associated haplotypes 3 and 4 were found in individuals of strain NA01-O established in 2013. A phylogenetic analysis of the 11 haplotypes obtained within this study suggested that haplotype 3, associated to deltamethrin resistance in bioassays, was closely related to susceptible haplotypes (1A, 1D-F, and 1H), but phylogenetically distant to the deltamethrin resistance-associated haplotypes 2 and 4 (Fig. 3.1). Further experiments focused on characterising the deltamethrin susceptibility phenotype of *L. salmonis* possessing haplotype 3, which differed from susceptibility-associated haplotypes 1A-H at only one of the 18 tested SNP loci, T8600C in COX1 (Table S3.6).

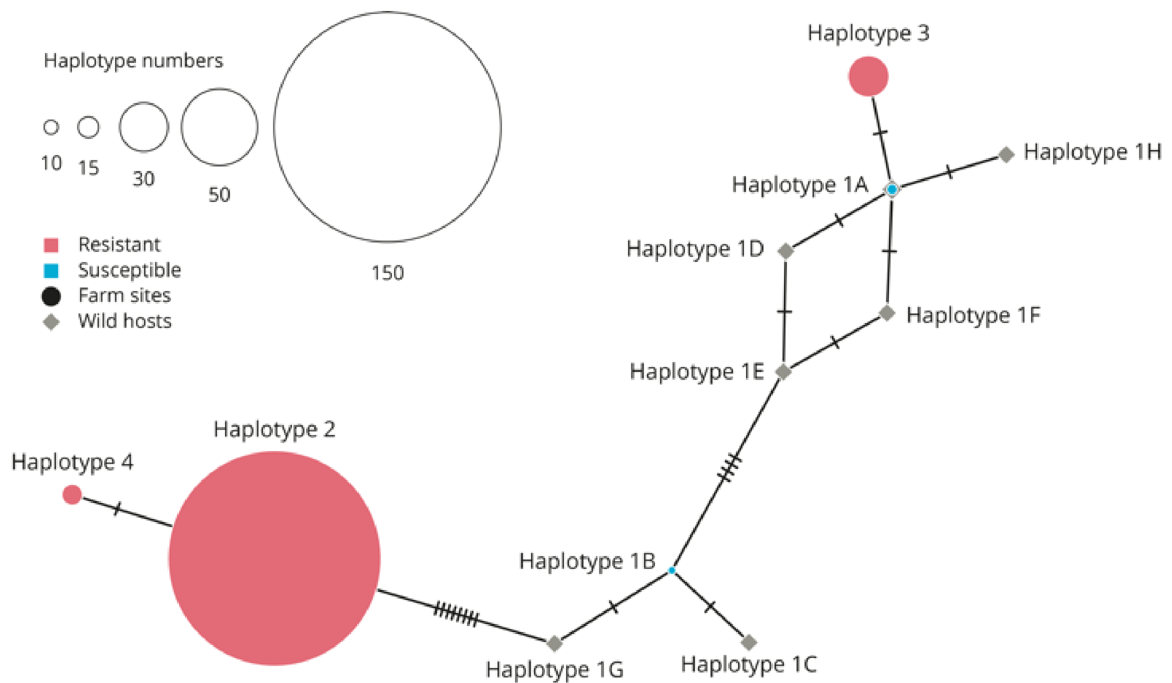

Figure 3.1 Median-joining haplotype network interfered from mitochondrial haplotypes of *L. salmonis*. Haplotypes were defined based on the combined occurrence of 18 mitochondrial SNPs, which have been associated with deltamethrin resistance in *L. salmonis* in a previous study (Carmona-Antoñanzas et al., 2017) (Tables S3.6 and S3.7). Haplotypes that were identified in salmon lice from farm sites are represented by circles, while haplotypes that were identified in salmon lice from wild hosts are represented by grey rhombi. The size of each circle is proportional to the frequency of each haplotype in salmon lice from farm sites. Deltamethrin resistance associated haplotypes are represented by red circles and susceptibility-associated haplotypes are shown in blue.

3.4.4 Establishment and characterisation of a laboratory strain with haplotype 3

The offspring of one field-collected gravid female with haplotype 3 were used to establish a new *L. salmonis* laboratory strain named IoA-10. The mitochondrial genome of IoA-10 was sequenced (EBI ENA project reference: PRJEB47839), revealing 36 strain-specific sequence variations that were absent in archived sequences from strains IoA-00, IoA-01, IoA-02, IoA-03, NA01-O, and NA01-P (Table S3.8). The mtDNA genome of IoA-10 strain lice was compared to that of previously analysed deltamethrin resistant, IoA-02, IoA-03, NA01-O, NA01-P, and deltamethrin susceptible *L. salmonis* strains, IoA-00, IoA-01 (Carmona-Antoñanzas et al., 2017), to identify SNPs associated with deltamethrin resistance. The non-synonymous mtDNA SNP T8600C, corresponding to Leu107Ser in COX1, and eight synonymous SNPs were the only mutations shared by all resistant and lacking in all susceptible parasites (Table 3.4).

Table 3.4 Sequence variations between *L. salmonis* from deltamethrin resistant (IoA-02, IoA-03, IoA-10, NA01-O, NA01-P) and deltamethrin susceptible (IoA-00, IoA-01) laboratory strains. Strain IoA-10 was sequenced within the present study, while strains IoA-02, IoA-03, NA01-O, NA01-P, IoA-00, and IoA-01 were sequenced by Carmona-Antoñanzas et al. (2017).

Position†	**Coverage (%)**	**Type**	**Location**	**Description**	**Original**	**Replaced by**
812	24	Polymorphism	D-loop		T	C
875	24	Polymorphism	D-loop		T	C
940	24	Deletion	D-loop		AG	A
960	24	Polymorphism	D-loop		T	C
963	24	Polymorphism	D-loop		G	A
972	24	Polymorphism	D-loop		A	G
8600	**42**	**Non-synonymous**	**COX1**	**TTG/Leu → TCG/Ser**	**T**	**C**
10178	30	Polymorphism	l-rRNA		A	G
15377	30	Polymorphism	D-loop		G	A

†Numbering according to the Scottish *L. salmonis* mitochondrial reference genome (NCBI accession no.: LT630766.1).

To assess the mode by which deltamethrin resistance of strain IoA-10 is passed on to the next generation, reciprocal crosses were performed between this strain and the drug-susceptible strain IoA-00 to produce two sets of F1 lice (Table S3.9). The deltamethrin susceptibility of parental and F1 populations was determined in bioassays (Table S3.11). Strains IoA-02 and IoA-10, as well as F1 offspring derived from IoA-10 dams and IoA-00 sires, were highly deltamethrin resistant (EC_{50} values > 24.0 µg L^{-1}; Fig. 3.2). In contrast, F1 animals derived from IoA-00 dams and IoA-10 sires (EC_{50} 0.55 µg L^{-1}; Fig. 3.2) were only marginally but significantly ($P < 0.0001$) less susceptible to deltamethrin than IoA-00 lice (EC_{50} 0.25 µg L^{-1}; Fig. 3.2).

Strain IoA-10 was only characterised regarding its resistance to deltamethrin. Thus, the possibility of multi-resistance against other compounds cannot be excluded.

To further characterise deltamethrin toxicity in IoA-10 lice, the effects of deltamethrin exposure on whole-body ATP levels in strain IoA-10 were investigated. Experiments further included fenpyroximate (100 µg L^{-1}), an acaricide known to block oxidative phosphorylation. As expected, fenpyroximate caused toxicity, and decreased whole-body ATP levels in all strains assessed (Fig. 3.3). Exposure to 2 µg L^{-1} deltamethrin caused toxic effects accompanied by significantly decrease of ATP levels in IoA-00 lice ($P = 0.008$), but failed at causing toxicity or significant effects on the ATP levels in IoA-02 and IoA-10 lice.

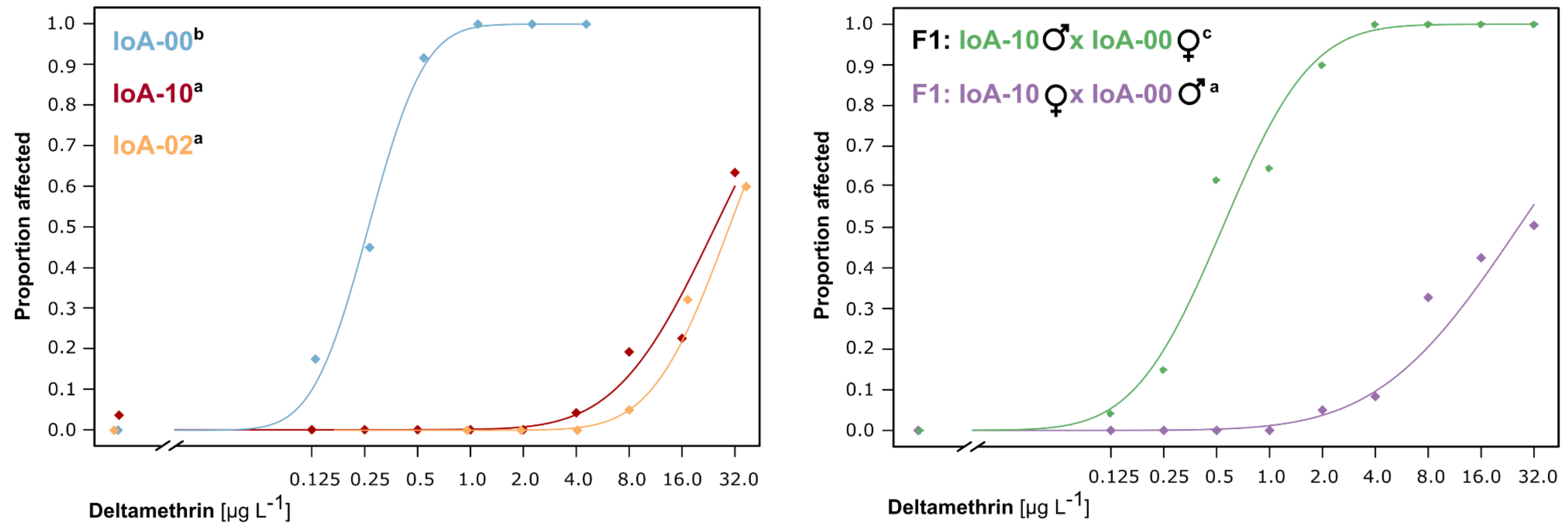

Figure 3.2 Standard deltamethrin bioassay with *L. salmonis* first filial (F1) progenies derived from parental (P0) crosses of different gender-strain orientations. Median effective concentrations EC_{50} [µg L^{-1}] and 95% confidence limits: IoA-10 = 24.73 (15.06-34.39), IoA-00 = 0.25 (0.20-0.30), IoA-02 = 25.95 (17.98-33.92), F1: IoA-10 dam x IoA-00 sire = 26.10 (11.46-40.75), F1: IoA-10 sire x IoA-00 dam = 0.55 (0.41-0.70). Bioassays involved exposure (30 min) to deltamethrin, followed by recovery in seawater (24 h) and rating of lice as normal or affected. Dose-response relationships were established for F1 females and males combined as sex differences were not significant ($P > 0.05$). Raw data are provided in Table S3.11. EC_{50} values with different letters are significantly different ($P < 0.05$).

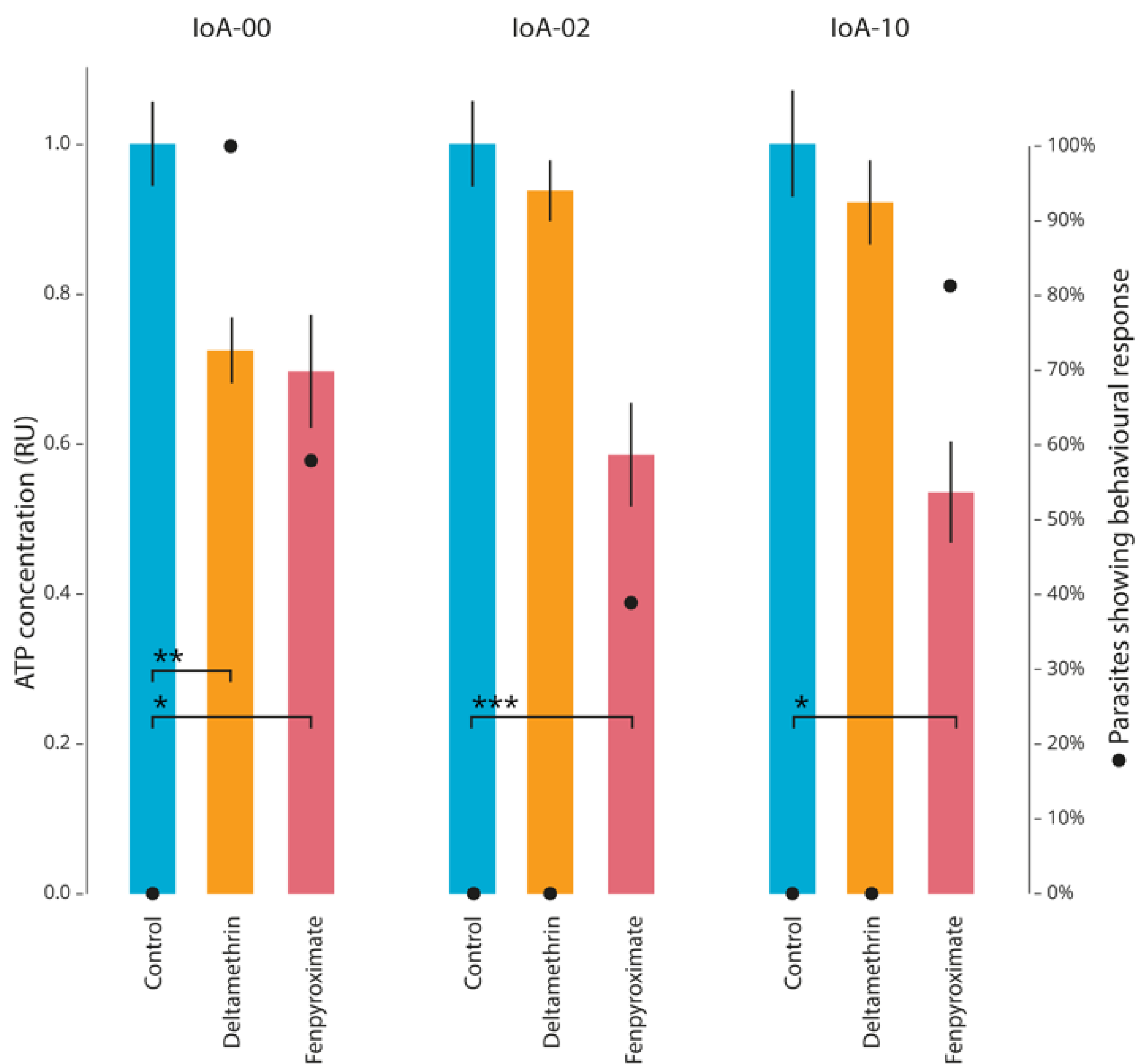

Figure 3.3 Effect of deltamethrin and fenpyroximate on ATP levels in *L. salmonis*. Male adult salmon lice of the drug susceptible strain IoA-00, the multi-resistant strain IoA-02, and strain IoA-10 were exposed to deltamethrin (2 µg L^{-1}), fenpyroximate (100 µg L^{-1}), or a solvent acetone control (0.05% v/v, control) for 300 min before behavioural effects were recorded (secondary y-axis; black data points) and alive animals were sampled for whole-body ATP analysis (n=15 per group). ATP concentrations in drug treated lice are expressed relative to those of the control group (relative units, RU ± SE). Stars indicate significant differences to the control group (Dunn's test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.4.5 Protein sequence comparisons of ND1, ND5, COX1, and COX3 among crustacean species

Amino acid sequences of ND1, ND5, COX1, and COX3 from *L. salmonis* and different crustacean species from diverse phylogenetic classes and habitats, for which mtDNA sequences were available for all four mitochondrial polypeptides, were aligned to reveal conserved residues. Among four non-synonymous mtDNA SNP loci that have previously been reported from a deltamethrin resistant strain (Carmona-Antoñanzas et al., 2017), G3338A (COX3 Gly33Glu), T5889C (ND5 Leu411Ser) and G8134A (ND1 Gly251Ser) cause residue changes at non-conserved positions, while T8600C (COX1 Leu107Ser) alters the amino acid sequence at a position that is conserved among all species assessed (Fig. S3.1).

3.5 Discussion

Previous studies have shown that deltamethrin resistance in *L. salmonis* is transmitted predominantly by maternal inheritance and associated with mtDNA SNPs (Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015). While an earlier study suggested that highly deltamethrin resistant *L. salmonis* share virtually identical mtDNA sequences (Carmona-Antoñanzas et al., 2017), the present study identified three mtDNA haplotypes associated with resistance, one of which coincides with the previously reported resistance associated mtDNA sequence. *L. salmonis* strain IoA-10, which possesses one of the novel deltamethrin resistance associated haplotypes, showed a high level of deltamethrin resistance comparable to that of the previously characterised strain IoA-02 and, similar to IoA-02, passed on deltamethrin resistance to the next generation through maternal inheritance. The phylogenetic analysis of mtDNA haplotypes provided evidence for multiple origins of mtDNA-associated deltamethrin resistance in *L. salmonis*. Comparison of mtDNA sequences between deltamethrin resistant and susceptible salmon louse strains suggested the association of deltamethrin resistance with SNP T8600C, corresponding to Leu107Ser in COX1.

L. salmonis obtained at aquaculture sites were subjected to bioassays to determine their deltamethrin susceptibility status. Parasites were then genotyped at previously described mtDNA SNP loci (Carmona-Antoñanzas et al., 2017) to identify mitochondrial haplotypes and assess haplotype association with deltamethrin resistance. 93%, 87%, and 100% of lice possessing haplotypes 2, 3, and 4, respectively, were classified as deltamethrin resistant, emphasising the association of mtDNA mutations with the resistance phenotype. The remaining 7-13% may have died due to interacting environmental factors or handling of parasites during sampling, transportation, and set-up of bioassays rather than drug toxicity (Aaen et al., 2015; Robertson et al., 2017). As bioassays were performed on lice directly obtained from farmed salmon, environmental conditions prior sampling could not be controlled. Lice may have also

been exposed to stressful conditions during sampling and due to abrupt changes of environmental conditions during transportation. The deltamethrin resistance phenotype was less distinct for lice containing haplotype 1. While most lice with this haplotype were classified as deltamethrin susceptible, 43% were rated resistant. The large number of resistant haplotype 1 individuals suggests the contribution of genetic factors other than mitochondrial mutations to the deltamethrin resistance phenotype, which is in line with findings by Carmona-Antoñanzas et al. (2017) who attributed the presence of about 20% resistant F2 parasites in a family descending from a deltamethrin susceptible dam and a resistant sire to nuclear genetic determinants of resistance.

To address confounding factors in bioassays on lice directly collected from farmed salmon, this study included analogous experiments with copepodid larvae derived from eggs of one female. When investigating effects of mtDNA mutations on drug resistance, advantages of F1 bioassays with copepodid larvae are that sibling clutches share the same mitochondrial haplotype (Scheffler, 2001) and were hatched and reared under standardised laboratory conditions less likely to bias the outcome of the experiment. While insufficient gravid females of appropriate genotype precluded analysis of haplotype 4 in copepodid bioassays, testing of larvae of the remaining haplotypes resolved significant differences in drug resistance between *L. salmonis* of haplotypes 2 and 3 as compared to parasites of haplotype 1, confirming results from conventional bioassays and underpinning the association of mtDNA mutations with deltamethrin resistance. Moreover, the study showed that deltamethrin resistance is already present in the larval stage, which would be expected for resistance conferred by mtDNA mutations (Carmona-Antoñanzas et al., 2017).

Genotyping of field isolates collected in 2018 and 2019 revealed three deltamethrin resistance-associated mtDNA haplotypes, with haplotypes 2, 3, and 4 being found in 75%, 14% and 8% of all resistant lice, respectively. Analysis of archived samples further provided evidence for haplotypes 4 being present in parasites removed from wild salmon in 2010, while haplotypes 3 and 4 were found in the deltamethrin resistant strain NA01-O, which was established in 2013. These findings contrast with results from Carmona-Antoñanzas et al. (2017), who reported mtDNA sequences consistent with haplotype 2 for all deltamethrin resistant isolates analysed in their study. Moreover, no resistance-associated haplotypes were evident from parasites collected from wild salmon in 2010. The failure to detect the less frequent haplotypes 3 and 4 in the earlier study (Carmona-Antoñanzas et al., 2017) could be attributed to the limited number of sequenced individuals.

Phylogenetic analyses of mtDNA haplotypes showed that the three deltamethrin resistance-associated haplotypes fall into two clusters, with haplotype 3 being phylogenetically distant to haplotypes 2 and 4, which shared 17 out of 18 tested SNP loci. This mtDNA sequence variability in deltamethrin resistant isolates indicate that mtDNA-associated deltamethrin resistance in *L. salmonis* originated from at least two independent origins. Thus, resistance-associated mitochondrial mutation(s) may have been selected for, more or less in parallel, as a consequence of the extensive use of pyrethroids.

Resistance-associated haplotypes 2 and 3 differed maximally in sequence, with only one out of 18 tested SNP loci being shared. To investigate the relationship of haplotypes 2 and 3 to deltamethrin resistance, the present study performed comparative experiments with strain IoA-02 containing haplotype 2 and strain IoA-10 containing haplotype 3. Despite haplotypes 2 and 3 being very different in sequence, their resistance phenotype is very similar. Both IoA-10 (haplotype 3) and IoA-02 (haplotype 2) lice were highly deltamethrin resistant (EC_{50} values >24.0 µg L^{-1}; $P = 0.845$) and reciprocal crosses of strains IoA-10 (present study) and IoA-02 (Carmona-Antoñanzas et al., 2017) with the drug-susceptible IoA-00 lice revealed that both strains transmit their resistance to the next generation through maternal inheritance. Moreover, deltamethrin exposure caused behavioural toxicity and whole-body ATP depletion in deltamethrin susceptible IoA-00 parasites, but not resistant IoA-10 and IoA-02 lice. These findings are in line with an earlier experiment, which compared the effect of deltamethrin exposure on behavioural toxicity and ATP levels between IoA-00 and IoA-02 lice (Carmona-Antoñanzas et al., 2017). The maternal inheritance of deltamethrin resistance in families derived from a resistant dam provide evidence that the resistance phenotype is conferred by the maternally transmitted mitochondrial genome, which has been discussed in detail elsewhere (Carmona-Antoñanzas et al., 2017). Depletion of ATP levels in deltamethrin susceptible lice may be related to the toxic effect of deltamethrin on the mitochondria, and mtDNA mutations in haplotypes 2 and 3 may have a protective effect.

As the inheritance of mtDNA is linear and lacks recombination through meiosis, relevant SNPs for deltamethrin resistance are transmitted together with irrelevant hitchhiking SNPs (Scheffler, 2001). Thus, SNPs that are truly linked to deltamethrin resistance are expected to be present in all resistance-associated haplotypes but lacking in all susceptibility-associated haplotypes. The non-synonymous mtDNA SNP T8600C, corresponding to Leu107Ser in COX1, was the only mutation shared by the resistance-associated haplotypes 2, 3, and 4 and lacking in all susceptibility-associated haplotypes. When comparing mtDNA sequences of deltamethrin resistant and susceptible lice, T8600C was also the only non-synonymous mutation differentiating between resistant and susceptible individuals. Sequencing analyses further

revealed eight additional SNPs in non-coding regions (NCR) of the mtDNA that were common to all resistant lice and lacking in all susceptible lice. NCR sequences are the most variable mtDNA sequences, which may explain the high number of SNPs found within this region in the present study (Nicholls and Minczuk, 2014). Seven of these SNPs were found in the mitochondrial control region, also known as displacement loop (D-loop). Its function is not yet fully understood but seems to be critical in regulating replication and transcription of mtDNA (Clayton, 1982). However, D-loop mutations are not known to confer drug resistance. Another SNP, A10178G, was found within a mitochondrial ribosomal RNA (rRNA) gene and has also been described by Bakke et al. (2018). Mitochondrial rRNAs are assembled with ribosomal proteins encoded by nuclear genes to form mitochondrial ribosomes, which are responsible for translating mitochondrial proteins (Sylvester et al., 2004). Thus, mutation within the mitochondrial rRNA may lead to ribosome dysfunction and may result in respiratory chain defects (Smith et al., 2014). However, to our knowledge, there are no reports of mitochondrial rRNA mutations associated with drug resistance.

Findings of the present study raise questions about the mechanism of deltamethrin resistance and by inference the mechanism of deltamethrin toxicity in *L. salmonis.* While it is generally accepted that pyrethroids target Na_v in terrestrial arthropods (Davies et al., 2007), several studies with terrestrial arthropods and mammals provide evidence for pyrethroid effects on mitochondrial functions. Due to their lipophilic nature, pyrethroids can pass and interact with biological membranes, making mitochondrial membranes and membrane proteins candidate targets for toxic action (Guven et al., 2018). For example, pyrethroids have been shown to affect mitochondrial membrane structures and dynamics, which can impair oxidative phosphorylation (Braguini et al., 2004; Gassner et al., 1997, Zhang et al., 2007). Mitochondrial oxidative phosphorylation can also be impaired by intracellular Ca^{2+} accumulation (Bauer and Murphy, 2020; Paschen, 2000), which can result from interactions of pyrethroids with Na_v and consequent Ca^{2+} influx (Hossain and Richardson, 2011) and direct effects of pyrethroids on voltage-gated Ca^{2+} channels (Clark and Symington, 2011).

Pyrethroid induced disruption of mitochondrial membrane integrity and inhibition of respiratory complexes, as well as intracellular Ca^{2+} accumulation can cause the generation of reactive oxygen species (ROS) in mitochondria (Brookes et al., 2004; Sipos et al., 2003; Truong et al., 2006). ROS can trigger a cascade of reactions that induces lipid peroxidation and damage of macromolecules (Chirico et al., 1993; Fang et al., 2002; Maiti et al., 1995). Pyrethroids have been shown to induce intrinsic mitochondrial apoptosis (Ko et al., 2016; Kumar et al., 2016), which involves mitochondrial outer membrane permeabilization, release of cytochrome C into the cytosol, activation of caspases, and ultimately DNA fragmentation (Bock and Tait, 2020). In particular,

this pathway can be triggered by pyrethroid induced oxidative stress and Ca^{2+} accumulation, as well as low levels of ATP that lead to disruption of the mitochondrial transmembrane potential (Buttke and Sandstrom, 1994; Hajnóczky et al., 2003; Kroemer, 2003; Lenaz, 1998). Interestingly, deltamethrin exposure increased apoptosis in mitochondria-rich skeletal muscle, subcuticular tissue, and central ganglion cells in salmon lice of a drug susceptible strain, but not or to a lesser degree in a deltamethrin resistant strain (Bakke et al., 2018).

Taken together, in salmon lice, deltamethrin may induce toxicity through disruption of mitochondrial membranes, direct inhibition of mitochondrial respiratory complex(es), intracellular Ca^{2+} accumulation, or by causing oxidative stress. An obvious explanation for deltamethrin toxicity in salmon lice would be that deltamethrin or its metabolites are binding to a mitochondrial respiratory complex and lead to disruption of the mitochondrial ATP production, which has been observed in the present study. In addition, inhibition of respiratory complexes can lead to the formation of ROS (Sipos et al., 2003). Both, low levels of ATP and oxidative stress can in turn induce intrinsic mitochondrial apoptosis (Buttke and Sandstrom, 1994; Kroemer, 2003; Lenaz et al., 2002), which has been described by Bakke et al. (2018). Resistance may be conferred by mitochondrial SNP(s) that changes the amino acid sequences of the complex and impair binding of deltamethrin. For reasons explained above, T8600C leading to Leu107Ser in COX1 is the most probable mutation for conferring deltamethrin resistance in salmon lice. Alternatively, deltamethrin might impair the mitochondrial ATP production in susceptible lice by causing disruptions of the mitochondrial membrane or by secondary effects arising from deltamethrin toxicity. In these scenarios, mtDNA mutation(s) may have functional effects on the efficiency of electron transfer or proton translocation, counteracting ATP deficits.

Conclusion

Deltamethrin resistance in *L. salmonis* is associated with multiple mtDNA haplotypes which have multiple origins. Non-synonymous mtDNA mutation T8600C, corresponding to Leu107Ser in COX1, was common to all deltamethrin resistant mtDNA haplotypes but lacking in haplotypes not associated with resistance. Parasites possessing a mtDNA haplotype in which T8600C was the only non-synonymous mutation are highly deltamethrin resistant and pass on their resistance to the next generation through maternal inheritance. The results suggest the association of deltamethrin resistance with SNP T8600C (Leu107Ser in COX1).

Chapter 4
Genomic analysis of the carboxylesterase family in the salmon louse (*Lepeophtheirus salmonis*)

4.1 Abstract

The pyrethroid deltamethrin and the macrocyclic lactone emamectin benzoate are used to treat infestations of farmed salmon by parasitic salmon lice, *Lepeophtheirus salmonis*. While the efficacy of both compounds against Atlantic populations of the parasite has decreased as a result of the evolution of resistance, the molecular mechanisms of drug resistance in *L. salmonis* are currently not fully understood. The functionally diverse CaE family includes members involved in pesticide resistance phenotypes of terrestrial arthropods. The present study had the objective to characterise the CaE family in *L. salmonis* and assess its role in drug resistance. *L. salmonis* CaE homologues were identified by homology searches in the parasite's transcriptome and genome. The transcript expression of CaEs predicted to be catalytically competent was studied using quantitative reverse-transcription PCR in drug susceptible and multi-resistant *L. salmonis*. The above strategy led to the identification of 21 CaEs genes/pseudogenes. Phylogenetic analyses assigned 13 CaEs to clades involved in neurodevelopmental signalling and cell adhesion, while three sequences were predicted to encode secreted enzymes. Ten CaEs were identified as being potentially catalytically competent. Transcript expression of acetylcholinesterase 1b (*ace1b*) was significantly increased in multi-resistant lice compared to drug-susceptible *L. salmonis*, with transcript abundance further increased in preadult-II females following emamectin benzoate exposure. In summary, results from the present study demonstrate that *L. salmonis* possesses fewer CaE gene family members than most arthropods characterised so far. Drug resistance in *L. salmonis* was associated with overexpression of *ace1b*.

4.2 Introduction

Sea lice of the family Caligidae (Copepoda) are ectoparasites of marine fish that feed on the mucus, skin, and blood of their hosts (Boxaspen, 2006). Depending on the severity of infections, sea lice can cause adverse effects in their fish hosts that include skin lesions, which are associated with a high risk of secondary infections, as well as osmoregulatory dysfunction, immunosuppression, increased stress, and reduced food conversion and growth rates (Grimnes and Jakobsen, 1996; Wootten et al., 1982). In 2018 the global costs of sea lice infestations to the salmon industry were estimated to exceed US $873 million/£700 million (Brooker et al., 2018b), comprising costs for prevention and treatments and, to a lesser extent, losses in production. In the Northern hemisphere, the salmon louse *Lepeophtheirus salmonis* is the major caligid species infecting salmonid fish (Costello, 2009). At salmon production sites, sea lice are controlled using

IPM combining veterinary drug treatments (Burridge et al., 2010) with a range of non-medicinal control approaches, which include mechanical and thermal delousing (reviewed by Holan et al., 2017) as well as the deployment of different species of cleaner fish that remove caligids from farmed salmon (Brooker et al., 2018a). Pharmaceuticals used for the control of sea lice are administered either orally as feed additives or topically as bath treatments. In-feed treatments include the macrocyclic lactone emamectin benzoate and different benzoylureas, while bath treatments include the organophosphate azamethiphos, the disinfectant hydrogen peroxide, and the pyrethroids cypermethrin and deltamethrin (Helgesen et al., 2019)

The continual use of a limited range of chemotherapeutants in pest control, with insufficient rotation between products of dissimilar mode of action, can lead to the evolution of resistance (Tabashnik et al., 2014). In treatment of *L. salmonis* infections, losses of efficacy have been reported for most available anti-parasitic drugs (Helgesen et al., 2019). In terrestrial arthropods, insecticide resistance most commonly involves one or both of two main molecular mechanisms. Resistance can result from mutations in genes coding for proteins constituting target sites of the pesticide (Williamson et al., 1993), or it can be based on enhanced detoxification by enzymes that break-down or sequester the pesticide (Ranson et al., 2002). Metabolic resistance typically involves members of large gene families with roles in detoxification, such as the CaEs, CYPs, GSTs, and ABC-transporter proteins.

Recent studies have identified molecular changes associated with pesticide resistance in *L. salmonis. L. salmonis* resistance to the organophosphate azamethiphos is primarily caused by a non-synonymous target-site mutation in a gene coding for AChE (Kaur et al., 2015b). Resistance of *L. salmonis* to the non-specific oxidant hydrogen peroxide has been linked to induction of catalase gene expression and enzymatic activity, as well as differential expression of five candidate genes including an aquaporin (Agusti-Ridaura et al., 2020). Deltamethrin resistance has been shown to be mainly inherited maternally and to be associated with mutations in the mtDNA (Carmona-Antoñanzas et al., 2017). In addition, a sodium channel mutation potentially further contributing to deltamethrin resistance has been identified (Carmona-Antoñanzas et al., 2019). Emamectin benzoate resistance has been linked to selective sweeps, with the genes under selection awaiting to be identified (Besnier et al., 2014). While the genomic complement of ABC transporters and CYPs in *L. salmonis* has been described (Carmona-Antoñanzas et al., 2015; Humble et al., 2019), existing studies do not provide evidence for an involvement of overexpression of members of these gene families in drug resistance in *L. salmonis* (Carmichael et al., 2013; Humble et al., 2019; Sutherland et al., 2015)

Esterases are a large group of metabolic enzymes that can be involved in resistance of arthropod pests to a wide range of chemical control agents, including pyrethroids and organophosphate esters (reviewed in Li et al., 2007). Most esterases involved in pesticide metabolism belong to the CaE gene family (Pfam PF00135 domain), a branch within the α/β-hydrolase fold superfamily (Pfam PF00561 domain) (Punta et al., 2012). The CaE family is functionally diverse. It comprises highly specialised enzymes acting on specific substrates, as well as less-selective enzymes with broad ranges of substrates, and catalytically inactive members with different roles including neurodevelopmental signalling or surface recognition (Oakeshott et al., 2005). Catalytically active CaEs possess a catalytic triad with a nucleophilic residue (serine (Ser), cysteine (Cys), or aspartate (Asp)), an acidic residue (glutamate (Glu) or Asp), and a histidine (His) residue (Myers et al., 1988). Some catalytically active CaEs catalyse the hydrolysis of ester pesticides, such as pyrethroids and organophosphates, into their corresponding acid and alcohol metabolites, which usually show low toxicity and are excreted readily. Furthermore, catalytically active CaEs have been shown to mediate resistance by sequestering ester and non-ester pesticides, impairing interactions with their toxicological target-sites (Hemingway, 2000). Esterase-mediated sequestration has, for example, been suggested to play an important role in resistance to the macrocyclic lactone spinosad (Herron et al., 2014)

In terrestrial arthropods, different molecular mechanisms of insecticide resistance involving esterases have been described (reviewed by Hemingway, 2000). Pesticide resistance can be based on the increased expression of esterases following gene amplification (Field and Devonshire, 1998; Rooker et al., 1996). Furthermore, single point mutations around the CaEs active site have been shown to induce organophosphate resistance by endowing the mutant enzyme with the ability to hydrolyse the pesticide (Campbell et al., 1998; Claudianos et al., 1999; Newcomb et al., 1997). In addition, constitutive upregulation of CaE gene expression has been implicated in pesticide resistance in several insect species (Zhu and Luttrell, 2015)

In *L. salmonis*, little is known about the CaE family and its potential roles in drug resistance. The aim of the present study was to identify members of the CaE family in *L. salmonis* and characterise their potential roles in resistance of the parasite to salmon delousing agents. Sequences encoding *L. salmonis* CaEs were isolated by homology searches of transcriptome and genome assemblies and annotated. Subsequently, CaE sequences were analysed *in silico* to identify proteins that are predicted to be catalytically competent and thus, have the potential to mediate pesticide resistance by hydrolysis or sequestration. Finally, potentially catalytically active CaEs were characterised regarding their transcript expression in two *L. salmonis* strains differing in susceptibility to delousing agents. The study further assessed the effects of sublethal

exposure to two salmon delousing agents, the pyrethroid deltamethrin and the macrocyclic lactone emamectin benzoate, on CaE transcript expression.

4.3 Materials and methods

4.3.1 Ethics statement

All research projects involving the UoS are subject to a thorough Ethical Review Process prior to any work being approved. The present research was assessed by the UoS AWERB and passed the ethical review process. Laboratory infections of Atlantic salmon with *L. salmonis* were performed under a valid UK Home Office license and at low parasite densities unlikely to compromise fish welfare.

4.3.2 Identification of *L. salmonis* CaE genes

L. salmonis CaE homologues were identified by tBLASTn searches in *L. salmonis* transcriptome (EBI ENA reference ERS237607) and genome assemblies (LSalAtl2s, metazoan.ensembl.org), using *D. melanogaster* CaEs (Oakeshott et al., 2005; Ranson et al., 2002) as queries (E-value cut-off = 10^{-10}; minimum alignment length of 40 amino acids; Table S4.1). NCBI accession numbers for *D. melanogaster* CaEs are compiled in Table S4.2. Each identified putative CaE locus was manually annotated using BlastP searches against the “non-redundant” sequence collection from the NCBI.

4.3.3 Phylogenetic analyses

Phylogenetic analyses of *L. salmonis* CaEs further took into account CaEs of *D. melanogaster* and *A. mellifera* (Claudianos et al., 2006) (NCBI accession numbers provided in Table S4.2). CaE amino acid sequences from *L. salmonis*, *D. melanogaster*, and *A. mellifera* and were aligned using default parameters in the online software MUSCLE version 3.8.31 (Multiple Sequence Comparison by Log-Expectation; https://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar, 2004). Model selection using the likelihood-based Akaike Information Criterion was performed with the online software SMS: Smart Model Selection in PhyML version 3.3.20200621 (http://www.atgc-montpellier.fr/phyml-sms/) (Lefort et al., 2017). A maximum likelihood phylogenetic tree was constructed using RAxML version 8.0 (Stamatakis, 2014) with a WAG matrix plus optimised invariable sites (+I), gamma distributed rate heterogeneity among sites (+G), amino acid frequencies estimated from the data (+F), and 1000 bootstrap replicates. The phylogenetic tree was visualised with FigTree version 1.4.4.

4.3.4 Prediction of protein function and subcellular localisation

L. salmonis CaE protein sequences were predicted from transcripts and analysed using InterPro version 79.0 (ebi.ac.uk/interpro/), an integrated documentation resource covering databases for protein families, domains, and functional sites (Jones et al., 2014). Additional active site motifs were identified from an alignment of *L. salmonis* CaE amino acid sequences with *D. melanogaster* acetylcholinesterase (DmAChE) (NCBI accession number 1QO9_A) using Clustal Omega version 2.1 (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers and Higgins, 2018). *L. salmonis* CaE sequences were predicted to encode catalytically competent enzymes if they contained the amino acid residues involved in the catalytic triad (Aranda et al., 2014), defined by serine, acidic (glutamate or aspartate) and histidine residues at positions corresponding to Ser238, Glu/Asp367, and His480 of the DmAChE sequence.

The program SignalP version 5.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict putative signal peptide sequences of *L. salmonis* CaEs to identify proteins secreted by the secretory pathway (Almagro Armenteros et al., 2019). Subcellular localisation of *L. salmonis* CaE proteins was assessed by DeepLoc version 1.0 (http://www.cbs.dtu.dk/services/DeepLoc/) (Almagro Armenteros et al., 2017).

4.3.5 *Lepeophtheirus. salmonis* strains and husbandry

Laboratory *L. salmonis* strains used in this study have been described in detail elsewhere (Carmona-Antoñanzas et al., 2016; Heumann et al., 2012). Strain IoA-00, which was taken into culture in 2003, is susceptible to deltamethrin, emamectin benzoate, and azamethiphos. Strain IoA-02 was established in 2011 and is multi-resistant, with resistance levels based on acute bioassays being 143-fold for deltamethrin, 4.3 to 7.3-fold for emamectin benzoate, and 23-fold for azamethiphos (Carmona-Antoñanzas et al., 2017, 2016; Humble et al., 2019).

L. salmonis strains were kept in culture at the Marine Environmental Research Laboratory of the University of Stirling (Machrihanish, UK). In brief, salmon lice were maintained on Atlantic salmon, which were held in circular tanks provided with a continuous supply of seawater and a photoperiod corresponding to natural day length. To propagate lines, egg strings obtained from gravid females were hatched and incubated to the infective copepodid stage, which were used to infect naïve Atlantic salmon. All laboratory infections were carried out under a valid UK Home Office license and at low parasite densities that were unlikely to compromise fish welfare. Infection trials were set up to produce preadult-II and adult parasites for chemical exposure experiments. Host fish were euthanised using a UK Home Office approved Schedule 1 method prior to the removal of salmon lice from fish.

4.3.6 Exposure of *L. salmonis* to deltamethrin and emamectin benzoate

L. salmonis adult males and preadult-II females of the drug susceptible strain IoA-00 and the multi-resistant strain IoA-02 were subjected to two concentrations of deltamethrin (0.05 µg L^{-1} and 2 µg L^{-1}) and emamectin benzoate (25 and 150 µg L^{-1}) (Pestanal® analytical standard grade, Sigma-Aldrich, Gillingham, UK) to elucidate potential effects of sublethal drug treatments on CaE transcript abundance.

L. salmonis were collected from host fish as described above and allowed to recover for 2 to 6 h in aerated seawater at 12°C. Individual parasites appearing viable based on attachment and swimming behaviour were randomly allocated to 300 mL crystallising dishes containing 100 mL of filtered (55 µm) seawater, with each dish receiving five preadult-II females and five adult males. Chemical exposures took place in a temperature-controlled chamber set to 12°C. Deltamethrin and emamectin benzoate were solubilised in PEG_{300} (M_n = 300). Chemical exposures were initiated by adding 50 µL of a 2000x final concentration solution of the relevant compound to crystallising dishes containing 100 mL seawater and salmon lice, resulting in a final solvent concentration of 0.05% (v/v) in all tests. No effects of PEG_{300} on transcript expression were detected in a previous microarray study (Carmichael et al., 2013).

Waterborne single exposures of *L. salmonis* involved a solvent control and two concentrations for each of the tested drugs (nominal concentrations: 0.05 µg L^{-1} and 2 µg L^{-1} deltamethrin; 25 and 150 µg L^{-1} emamectin benzoate). All drug treatments were expected to be sublethal to IoA-02, while the higher concentration of each drug was expected to be lethal to IoA-00 (Carmona-Antoñanzas et al., 2016; Heumann et al., 2012). In previous studies using the same bioassay methodology, measured drug concentrations in bioassays were 68 to 133% of nominal concentrations for deltamethrin, and 50% of nominal concentrations for emamectin benzoate (Carmichael et al., 2013, Carmona-Antoñanzas et al., 2017). Reflecting recommended conditions for *L. salmonis* bioassays (SEARCH Consortium, 2006), parasites were exposed to deltamethrin for 30 min and then transferred to clean seawater for 24 h recovery, while exposures to emamectin benzoate were for 24 h. Subsequently, the behavioural responses of test individuals were examined and rated. Rating criteria based on observed behavioural responses (live, weak, moribund, dead) have been described in detail elsewhere (Carmona-Antoñanzas et al., 2016). Parasites rated as “live” or “weak” were considered unaffected, while “moribund” and “dead” parasites were considered affected. Only individuals deemed unaffected were collected for RNA extraction and subsequent determination of transcript abundance. Parasites were sampled in RNA stabilisation solution (4.54 M ammonium sulphate, 25 mM trisodium citrate, 20 mM EDTA, pH 5.4), stored overnight at 4°C, and transferred to nuclease-free tubes for storage at -70°C pending RNA extraction.

4.3.7 RNA extraction and cDNA synthesis

Individual salmon lice were homogenised in 1 mL TRI Reagent® (Sigma-Aldrich, Gillingham, UK) using a bead-beater homogeniser (BioSpec, Bartlesville, OK, USA) and total RNA was extracted following the manufacturer's instructions. After phase separation, RNA was precipitated from the aqueous phase by adding 0.5 volumes of 2-propanol and 0.5 volumes of high salt buffer (0.8 M sodium citrate sesquihydrate; 1.2 M sodium chloride). Total RNA was resuspended in nuclease-free water (15 µL for adult males and 20 µL for preadult-II females). Quantity and quality of isolated total RNA were determined by UV spectrophotometry using a ND-1000 NanoDrop® (Thermo Scientific, Hemel Hempstead, UK) and RNA integrity was assessed by electrophoresis using 250 ng of denaturised total RNA in a 1% agarose gel stained with ethidium bromide. For each salmon louse, 2 µg total RNA was treated with 2 U DNase (DNA-*free*™ Kit, Ambion®, Kaufungen, Germany) following the manufacturer's instructions. 2 µg DNA free total RNA of each sample were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) without RNase inhibitor, according to the manufacturers protocol. Reverse transcriptions were carried out including negative controls omitting RNA (NTC) and controls containing no enzyme (RT-). All cDNA samples were stored at -70°C for further use.

4.3.8 Quantitative expression analyses by reverse transcription-quantitative PCR (RT-qPCR)

L. salmonis CaEs that contained an intact catalytic triad (see section 4.3.4) and/or grouped into clades of high bootstrap support with *D. melanogaster*, *A. mellifera*, or *L. salmonis* CaE sequences with a conserved catalytic triad (see section 4.3.3) were classified as potentially catalytically competent. As catalytically competent CaEs have the potential to mediate pesticide resistance by hydrolysis or sequestration, only potentially catalytically competent CaEs were selected for RT-qPCR studies. Six male and six female parasites were analysed for each combination of treatment and strain. Five reference genes (ribosomal subunit 40S, *40S*; ribosomal subunit 60S, *60S*; elongation factor 1-alpha, *efa*; hypoxanthine-guanine phosphoribosyltransferase, *hgprt*; and RMD-5 homologue) were quantified and *40S* (M stability value = 0.244), *60S* (M stability value = 0.257), and *efa* (M stability value = 0.244) selected as reference genes as being most stable in *L. salmonis* according to GeNorm (Vandesompele et al., 2002).

The relative transcript expression of target and reference genes was measured by RT-qPCR using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates. Primer sequences are provided in Table S4.3. Each sample was analysed in duplicate 10 µL reaction volumes containing 5 µL Luminaris Colour Highreen qPCR Mix (Thermo Scientific, Hemel

Hempstead, UK), 0.5 µL (10 pmol) each for the forward and reverse primer, 2.5 µL of 20-fold diluted cDNA for the target genes or 1 µL of 20-fold diluted cDNA for the reference genes and nuclease-free water. Each qPCR run was comprised of an activation step (50°C for 2 min), then initial denaturation (95°C for 15 min), followed by 35 cycles of denaturation, annealing, and extension (15 s at 95°C, 30 s at the primer pair specific annealing temperature (Table S4.3), and 30 s at 72°C). Finally, a melting curve with 1°C increments during 6 s from 60 to 95°C was performed to check the presence of a single product in each reaction. Control reactions included NTC and RT-.

For each RT-qPCR run, a standard curve was generated from a parallel set of reactions containing serial dilutions (1/5, 1/10, 1/20, 1/50, 1/100, 1/200, 1/500) of a cDNA pool derived from the samples. Standard curves were used to evaluate the efficiency of the primers, melting curves, and cycle threshold (Ct) values, and the combined efficiency of the primers and assay (Larionov et al., 2005). Primers used showed efficiencies in the range between 0.80 and 1.10 and resulted in amplifications characterised by a single melting peak and cycle threshold (Ct) values below 30. Ct values, melting curves, standard curves, and primer efficiencies were calculated by linked PCR cycler software (qPCR Soft 4.0). The size of the amplified qPCR product was checked by agarose gel electrophoresis along with appropriate markers and the reaction specificity was confirmed by sequencing the qPCR amplicon.

Relative transcript quantification was achieved by including on each PCR plate a parallel set of serial dilutions of a pool of all experimental cDNA samples, allowing derivation of the estimated relative copy number of the transcript of interest for each sample, corrected for the efficiency of the reaction. The normalised expression values (relative units, RUs) were generated by the ΔCt method (Pfaffl, 2001) with results expressed as the ratio between the estimated relative copy number of the target genes and a reference gene index calculated from the geometric mean of the estimated relative copy number of the three most stable reference genes *40S*, *60S* and *efa*.

4.3.9 Sequencing of *L. salmonis* CaE genes

L. salmonis CaE sequences that were predicted to be potentially catalytically competent were subjected to rapid amplification of 5’ and 3’ cDNA ends (RACE) to obtain their complete open-reading frame (Table S4.4). 5’ and 3’ RACE was carried out using the SMARTer RACE 5’/3’ Kit (Takara Bio, CA, USA) according to the manufacturer's protocol, using Q5® High-Fidelity 2x Master Mix (New England BioLabs Ltd, Hitchin, UK). under the following conditions: 98°C for 30s, 5 cycles of 98°C for 10 s and 72°C for 1 min, then 5 cycles of 98°C for 10s, 70°C for 30s and 72°C for 1 min, followed by 25 cycles of 98°C for 10s, 68°C for 30s and 72°C for 1 min, and a final

extension at 72°C for 2 min. RACE products were separated by 1% agarose gel electrophoresis, purified and subcloned (pGEM-T Easy Vector system and *Escherichia coli* JM-109, Promega, WI, USA). Plasmids were isolated and inserts subjected to Sanger sequencing using a commercial service. The 5' and 3' amplicons and their associated CaE cDNA transcripts from the NCBI Nucleotide and EnsemblMetazoa databases were assembled using the software SeqMan Pro (DNASTAR, WI, USA). To confirm the assembly, each cDNA sequence was amplified in one PCR, subcloned, and sequenced (Table S4.4), as described above. Sequences obtained for the same PCR products were aligned to obtain contiguous cDNA sequences, which were deposed in the European Nucleotide Archive [project PRJEB40940] (see Table S4.4 for accession numbers).

4.3.10 Single nucleotide polymorphisms (SNPs) in CaE genes

To identify and analyse SNPs in CaE genes predicted to be catalytically competent, available RNA-seq data for strains IoA-00 ($N_{\text{Sequenced animals}}$=8) and IoA-02 ($N_{\text{Sequenced animals}}$=7) were used (ENA Project accession PRJEB41730). Using the hisat2 version 2.2.1 (Kim et al., 2019), sequencing reads were aligned to *L. salmonis* CaE cDNA sequences. Sequence variations were identified using the HaplotypeCaller function in GATK version 4.2.0.0 (Poplin et al., 2018).

4.3.11 Statistical Analyses

Relative CaE expression data were tested for normality and homogeneity of variance using the Shapiro-Wilk's test and the Levene's test, respectively. As some data sets violated these homoscedasticity assumptions, non-parametric tests were employed in further analyses, performed in R version 3.5.0 (packages car, rcompanion, PMCMR). Effects of *L. salmonis* strain and sex/stage on CaE transcript expression were determined using the Scheirer-Ray-Hare test. The Kruskal-Wallis test was used to assess the effect of drug treatments on transcript expression. To account for the simultaneous testing of ten transcripts and control the experiment-wise type I error, sequential Bonferroni correction was applied (Rice, 1989). After significant Kruskal-Wallis tests, Dunn's test was employed for post-hoc comparisons to the control group. Statistically significant expression differences between groups were considered biologically significant when exceeding the between-group difference of the estimated relative reference gene expression. In analyses of SNP expression between strains IoA-00 and IoA-02, genotype frequencies at each polymorphic site were compared using the Fisher's exact probability test, using the program Genepop version 4.7.5 (https://genepop.curtin.edu.au/) (Raymond, 1995; Raymond and Rousset, 1995; Rousset, 2008). The significance level was set at $P < 0.05$ in all tests.

4.4 Results

4.4.1 Identification of *L. salmonis* CaEs

L. salmonis CaEs were identified by homology searches in a reference transcriptome (EBI ENA reference ERS237607) and a genome assembly (LSalAtl2s, metazoan.ensembl.org) of the species. Of a total of 21 putative CaE genes/pseudogenes identified in the genome, 20 had matching transcripts (Table S4.1), with three gene models being represented by more than one transcript. While eight of the CaE sequences identified were partial, all *L. salmonis* CaE sequences lacked disabling frameshifts and in-frame stop codons.

4.4.2 Phylogenetic analyses and classification

L. salmonis CaEs were subjected to phylogenetic analyses together with CaE sequences of *D. melanogaster* and *A. mellifera* (Fig. 4.1). The observed phylogenetic topology conforms to the phylogenetic classification scheme proposed by Oakeshott et al. (2005), who divided the CaE family into 14 clades (A-N) nested within three functional classes, with classes 1 to 3 being defined as the dietary/detoxification, the hormone/semiochemical processing, and the neuro/developmental classes, respectively. The 21 identified *L. salmonis* CaEs grouped into seven clades within two classes. The third class showed 13 *L. salmonis* members, which assigned to clades J (AChE); n=2), K (gliotactins; n=1), L (neuroligins; n=6), M (neurotactins=2), and I (uncharacterised proteins, n=2), while the second class contained three members clustering into clades H (glutactins; n=2) and E (secreted β esterases; n=1). Five CaEs clustered together in a novel clade (clade O). BLAST annotation of *L. salmonis* CaEs confirmed the classification of sequences assigned to clades J to M as AChEs, gliotactins, neuroligins and neurotactins, respectively (Table S4.1). The two AChEs found in this study (HACA01023258.1, HACA01002875.1) have been described previously (Kaur et al., 2015a)

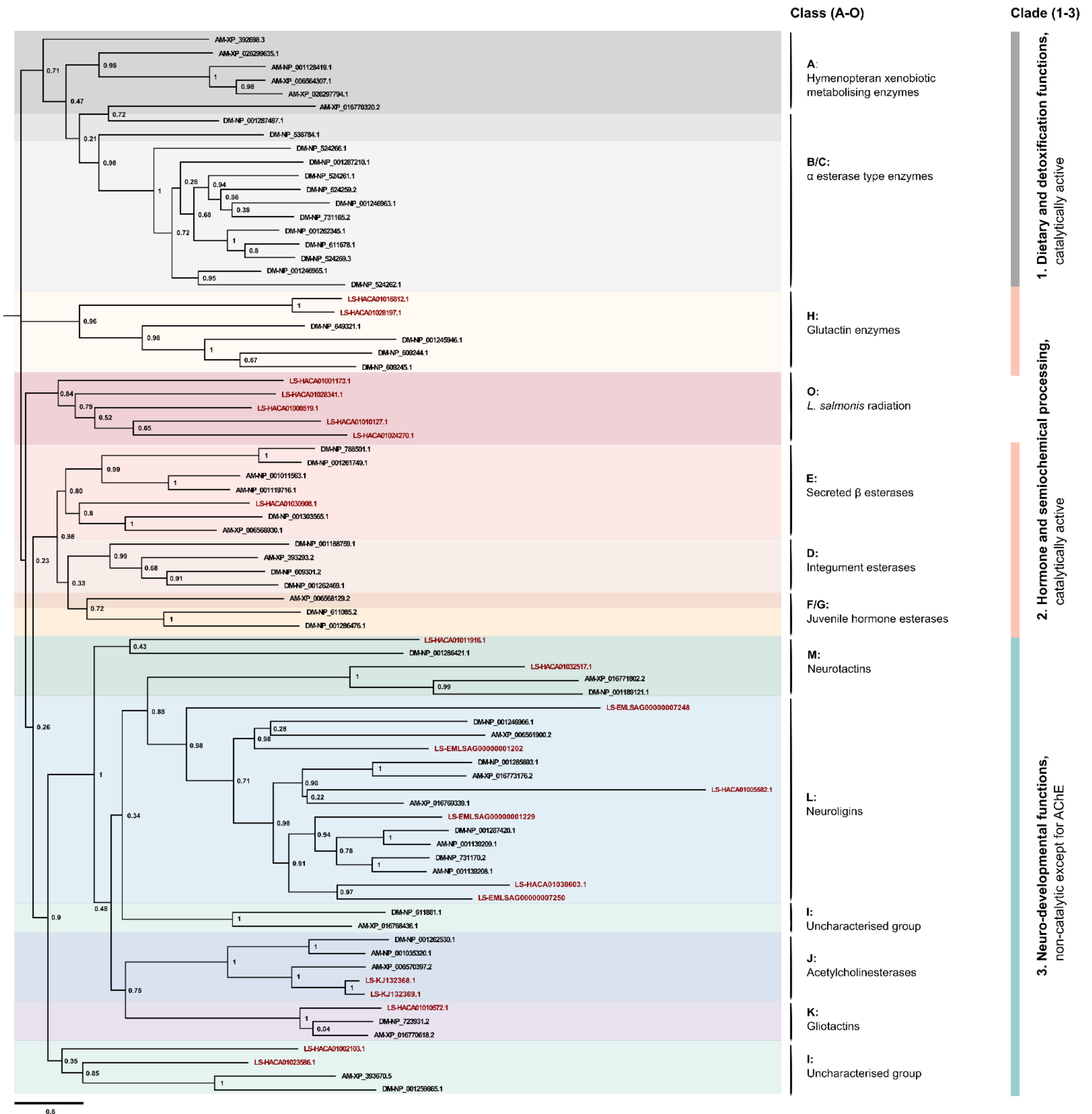

Figure 4.1 Phylogenetic relationship of carboxylesterases (CaEs) in *L. salmonis*, *D. melanogaster*, and *A. mellifera*. The alignment was constructed using Multiple Sequence Comparison by Log-Expectation (MUSCLE) and phylogenetic relationship was conducted by Maximum likelihood (ML) analysis using RaxML. ML bootstrap support values (BS) (percentage of 1000 BS) are provided next to the nodes. *L. salmonis* (LS) CaEs are highlighted in red. DM: *D. melanogaster*. AM: *A. mellifera*.

4.4.3 Conserved domains and predicted subcellular localization

In silico analyses confirmed that the identified *L. salmonis* sequences were CaEs possessing the Pfam PF00135 domain (Fig. 4.2) (Punta et al., 2012). Amino acid alignment of *L. salmonis* CaEs with *D. melanogaster* DmAChE revealed that seven *L. salmonis* sequences contained the amino acid motif of the catalytic triad, consisting of Ser, Glu or Asp and His residues, as well as amino acid residues constituting the active site, including the nucleophilic elbow (GXSXG), the oxyanion hole (GG), and a highly conserved Ser residue (Fig. 4.2). CaEs showing these features included all members of clade H within class 2, three members of the new clade O, and the two *L. salmonis* AChE (HACA01023258.1, HACA01002875.1) assigned to clade J in class 3. Three CaE sequences within clades O and E lacked catalytic triad residues but grouped in clusters of high bootstraps-support with *D. melanogaster*, *A. mellifera,* or *L. salmonis* CaE sequences with a conserved catalytic triad (Fig. 4.1, Fig. S4.1). Further bioinformatic analyses predicted members of clades K (gliotactins) and L (neuroligins) to be membrane associated (Table S4.5). In contrast, all members of the class 2 (clades H and E) were predicted to be soluble and secreted. Similarly, CaE sequences assigned to clade O were predicted to be soluble, possessing either a cytoplasmic or an endoplasmic reticulum targeting signal (Table S4.5).

Class	Clade	Accession no.	Superfamily[++] α/β hydrolase PF00561 IPR029058	Family[++] CaE Type B PF00135 IPR002018	Disulfide C	Disulfide C	Active site residues: Oxyanion hole GG	Active site residues / Catalytic triad: Serine residue GXSXG[+]	Serine residue S	Disulfide C	Disulfide C	Catalytic triad: Acidic residue E or D	Catalytic triad: Histidine residue H
					66	93	149, 150	238	264	292	307	367	480
	J	**DmAChE 1QO9_A**[†]	✓	✓	ATCVQE	EDCLYI	WIYGGGFM	GESAGS	MQSGT	CNCNA	MSCMR	RDEGTY	VLHGDE
2	**H**	HACA01028197.1[†,‡]	✓	✓	PLCPQG	EDCLHL	FIHGGGFS	GESAGS	GQSGS	LGCKT	VKCLR	LYEGIY	ACHADE
	H	HACA01016812.1[†,‡]	✓	✓	PVCPQG	EDCLHL	FVHGGGFS	GESAGS	GQSGS	LGCKT	VKCLR	LYEGIY	ACHADE
	O	HACA01024270.1[†,‡]	✓	✓	KMGYQP	DDCLYL	YFHGGAFI	GQSAGG	CLSGA	LGVNV	LTQLR	AQDGLS	AAHGDE
	O	HACA01001173.1[†,‡]	✓	✓	PACPQQ	EDCLYL	WIHGGNFM	GESSGA	IQSGS	MGCI-	QECIQ	SNEGFL	ASHADE
	O	HACA01008519.1[†,‡]	✓	✓	HICPQY	EDCLFL	FIHGGGFK	GSSAGG	SQSTP	MGC--	SKCLK	SEEGAM	VLHGDE
	O	HACA01010127.1[†,‡]	✓	✓	------	------	--------	------	-----	-----	-----	RHSGIA	ISHWDE
	O	HACA01028341.1[†,‡]	✓	✓	------	------	--------	GHGSGA	TQSGS	VGCT-	LKCLR	AEEGML	TCHGDE
	E	HACA01030908.1[†,‡]	✓	✓	HFCPQH	EDCLWL	WIHGGNFV	GQQAGG	SLSGS	LECPY	IECIR	DDEGAF	VGNGDD
	J	HACA01002875.1[†]	✓	✓	NSCIQV	EDCLYL	WIYGGGFY	GESAGG	MQSSS	MRCPY	IECLL	KDEGNF	VLHGDE
	J	HACA01023258.1[†]	✓	✓	NSCIQV	EDCLYL	WIYGGGFY	GESAGG	MQSAS	MSCPY	IECLR	KEEGNY	VLHGDE
	I	HACA01002103.1[†]	✓	✓	PACPQE	ENCLW	FLHPPHWD	GHGSGG	SMSGS	-----	-----	SEEGKL	LSHGDE
	I	HACA01023586.1[†]	✓	✓	HICPQY	EDCLFL	HIHGGAFI	GEDAGA	ALSGN	LECSS	IECIS	KNGGAF	VVHGDE
	K	HACA01010572.1[†]	✓	✓	------	ENCLFL	YIHGGEFQ	GPGAGG	SMSGS	VGCTI	VDCLR	KDDAAY	ISHNLE
	L	HACA01030603.1[†]	✓	✓	------	------	--------	------	-MSGS	LNCTI	ITCLR	EDETNN	SQHGSM
3	**L**	EMLSAG00000007248[§]	✓	✓	PVCPQK	EDCLYL	--------	------	-----	-QCSQ	-----	------	------
	L	EMSLAG00000007250[§]	✓	✓	------	------	YVHGESFK	GHGTG-	LMSGS	FDCIE	ISCLR	THDYFN	STHGSE
	L	EMSLAG00000001202[§]	✓	✓	------	------	--------	GHGTGA	LMSGS	LNCSA	LSCLR	TADALF	CAHGEE
	L	EMSLAG00000001229[§]	✓	✓	------	------	--------	------	MMSGS	LRCPL	MNCLR	SSEAFH	SIHGEE
	L	HACA01005582.1[†]	✓	✓	PVCPQL	EDCLYL	--------	------	-----	-----	-----	------	------
	M	HACA01032517.1[†]	✓	✓	------	------	YIRGDDES	GSGFGA	WVSNG	LFCGP	ERCLI	EHV---	-AHSDI
	M	HACA01011916.1[†]	✓	✓	PACSQI	EDCLYL	WIHGGDFS	GSGAGG	SSSGI	LSCPT	KSCLS	KYDENL	TKYGGE

Figure 4.2 Conserved motifs in L. salmonis carboxylesterase (CaE) sequences. *L. salmonis* CaE sequences were aligned against the reference *Drosophila melanogaster* acetylcholine esterase (DmAChE) sequence. Amino acid residues were numbered according to DmAChE. Conserved catalytic triad residues (Ser238, Glu/Asp367, and His480) are shown in green. Additional amino acid residues within the active site (oxyanion hole G149 and G150, putative catalytic tetrad residue Ser264 (Thomas et al., 1999) are shown in blue. Conserved disulphide bridges (Cys66, Cys98 and Cys292, Cys307) are shown in yellow. "-" indicates a gap in the alignment. [†]NCBI accession number. [‡]RT-PCR followed by Sanger sequencing was used to confirm cDNA sequences, which were deposited in the European Nucleotide Archive (see Table S4.4 for accession numbers). [§]EnsemblMetazoa accession number. [+]GXSXG: Nucleophilic elbow. [++]Family affiliation according to Pfam (PF) and InterPro (IPR) entries. The CaE family type B belongs to the superfamily α/β hydrolase fold (PF00561, IPR029058).

4.4.4 Transcript expression of *L. salmonis* CaEs

Ten *L. salmonis* CaEs, which were predicted to be catalytically competent based on phylogenetic and protein functional analyses, were selected to study their transcript expression using qPCR.

The assessment of CaE transcript abundance in preadult-II females and adult males of the drug susceptible strain IoA-00 and the multi-resistant strain IoA-02 revealed significant effects of parasite sex/stage on transcript expression. As the estimated relative reference gene expression was found to be 2.14-fold larger in preadult-II females than in adult males (Table S4.6), only effects of sex/stage larger than 2.14-fold were considered biologically significant. Applying this threshold, five out of ten tested CaEs (HACA01023258.1, HACA01030908.1, HACA01028197.1, HACA01016812.1, HACA01010127.1) showed significant sex/stage-biased transcript expression (Table 4.1).

In addition, transcript abundance of HACA01002875.1 (clade J, *ace1b*) and HACA01010127.1 (clade O) was significantly increased in strain IoA-02 compared to strain IoA-00 ($P < 0.01$) (Table 4.1). The effects of drug exposure were studied for the pyrethroid deltamethrin (Fig. 4.3) and the macrocyclic lactone emamectin benzoate (Fig. 4.4). Parasites of strains IoA-00 and IoA-02 were exposed to low sublethal concentrations of the compounds (0.05 µg L^{-1} deltamethrin; 25 µg L^{-1} emamectin benzoate), as well as higher concentrations (25 µg L^{-1} deltamethrin; 150 µg L^{-1} emamectin benzoate) that were tolerated by IoA-02 animals but lethal for IoA-00 parasites, with no survivors available for transcript expression studies (Table S4.7). Compared to transcript levels in untreated control parasites, transcript expression of HACA01002875.1 (clade J, *ace1b*) was significantly increased ($P < 0.05$) in IoA-00 preadult-II females after treatment with 25 µg L^{-1} emamectin benzoate and in IoA-02 preadult-II females after treatment with 150 µg L^{-1} emamectin benzoate (Table 4.2).

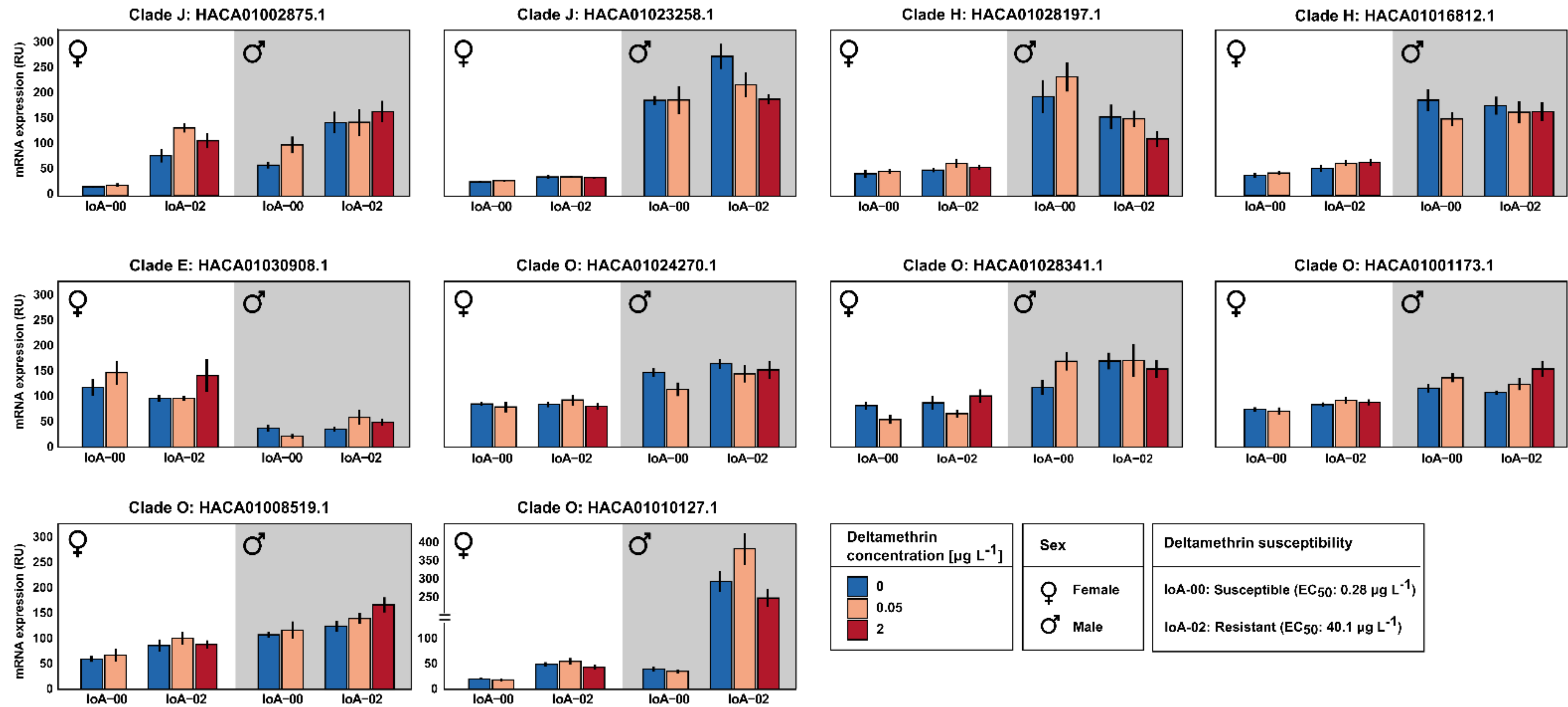

Figure 4.3 Effect of deltamethrin exposure on carboxylesterase (CaE) transcript expression in *L. salmonis*. Preadult-II females and adult males of the drug susceptible strain IoA-00 and the multi-resistant strain IoA-02 were exposed to deltamethrin (0.05 μg L^{-1}; 2.0 μg L^{-1}) for 30 min and allowed to recover for 24 h in seawater before esterase transcript expression was determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Gene expression was expressed as relative units (RUs) calculated from the mean normalised ratios (n = 6 ± SE) between the estimated relative copy numbers of target genes and the estimated relative copy numbers of the reference genes. Bars bearing stars are significantly different (Dunn's test post-hoc comparisons to the control group).

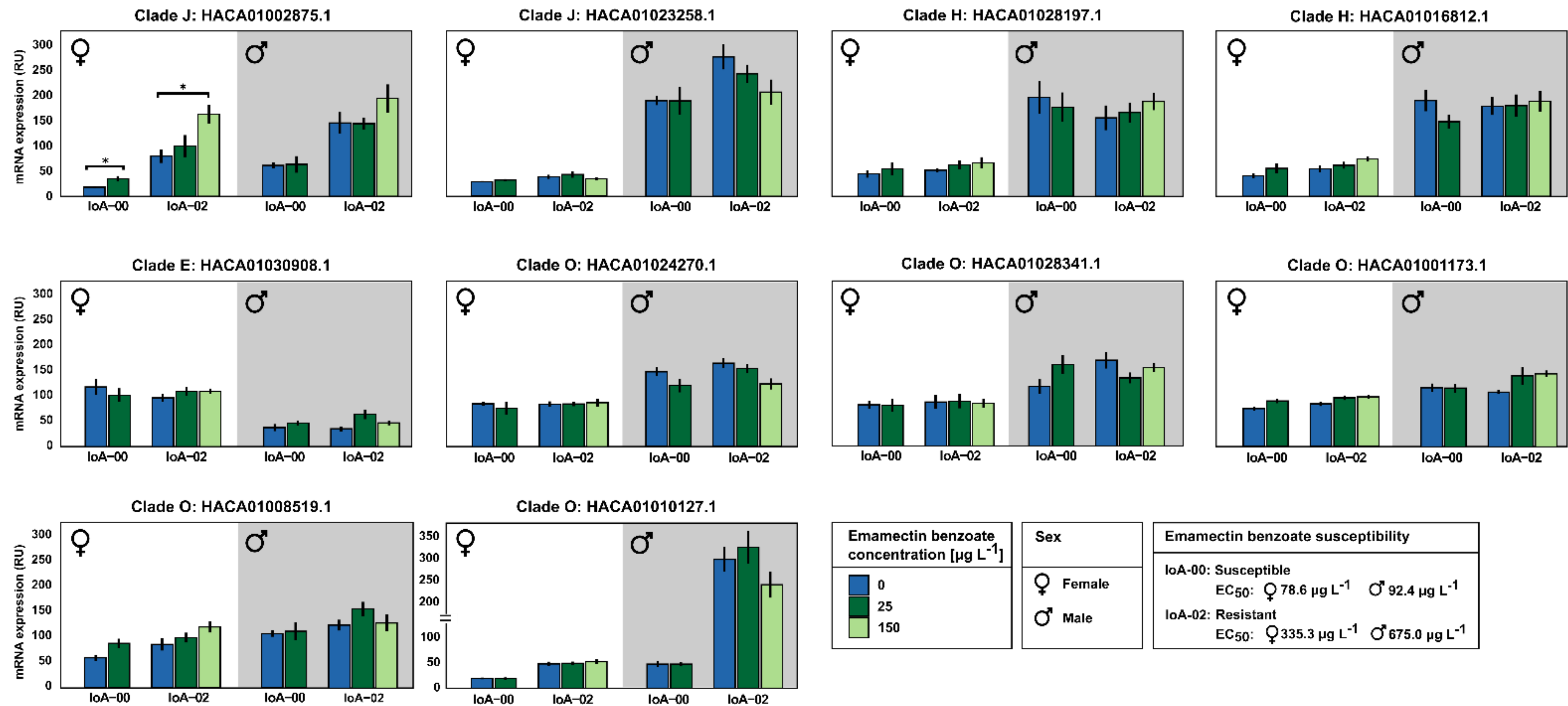

Figure 4.4 Effect of emamectin benzoate exposure on carboxylesterase (CaE) transcript expression in *L. salmonis*. Preadult-II females and adult males of the drug susceptible strain IoA-00 and the multi-resistant strain IoA-02 were exposed to deltamethrin (25 µg L^{-1}; 150 µg L^{-1}) for 30 min and allowed to recover for 24 h in seawater before esterase transcript expression was determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Gene expression was expressed as relative units (RUs) calculated from the mean normalised ratios (n = 6 ± SE) between the estimated relative copy numbers of target genes and the estimated relative copy numbers of the reference genes. Bars bearing stars are significantly different (Dunn's test post-hoc comparisons to the control group; *significant at $P < 0.05$, **significant at $P < 0.01$).

Table 4.1 Carboxylesterase (CaE) transcript expression in two *L. salmonis* strains differing in drug susceptibility. Transcript expression of CaEs was determined by quantitative reverse transcription polymerase chain reaction in preadult-II females and adult males of the drug susceptible strain IoA-00 and the multi-resistant strain IoA-02. Effects of strain, sex/stage, and interaction of strain and sex/stage were assessed by the Scheirer-Ray-Hare test.

Clade	NCBI accession no.	p-value Strain	Fold change Strain	*P*-value Sex/Stage	Fold change Sex/Stage	*P*-value Strain* Sex/Stage
J	HACA01002875.1	0.0011**	2.84	0.012*	2.12	0.742
J	HACA01023258.1	0.094	1.44	0.0001***	7.0	0.905
E	HACA01030908.1	0.549	1.18	0.0001***	2.93	0.936
H	HACA01028197.1	0.908	1.16	0.0001***	3.66	0.564
H	HACA01016812.1	0.577	1.01	0.0001***	3.90	0.565
O	HACA01024270.1	0.805	1.07	0.0001***	1.85	0.613
O	HACA01010127.1	0.009**	4.57	0.0001***	5.09	0.90
O	HACA01001173.1	0.644	1.00	0.0001***	1.41	0.488
O	HACA01028341.1	0.235	1.29	0.0023**	1.70	0.332
O	HACA01008519.1	0.133	1.26	0.001***	1.60	0.686

*Significant at $P < 0.05$; **Significant at $P < 0.01$; ***Significant at $P < 0.001$.

Table 4.2 Effect of chemical treatments on carboxylesterase (CaE) transcript expression in *L. salmonis*. Transcript expression of CaEs was determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) in preadult-II females and adult males of the drug susceptible strain IoA-00 and the multi-resistant strain IoA-02. Parasites were exposed to deltamethrin (0.05 µg L^{-1}, 2.0 µg L^{-1}) or emamectin benzoate (25 µg L^{-1}, 150 µg L^{-1}). For each strain, the CaE transcript expression was compared among chemical treatments and untreated controls using the Kruskal-Wallis test. The Dunn's test was employed for post-hoc comparisons of chemical treatments to the control group (see Fig. 4.3 and 4.4). The experimental-wise type I error was controlled by sequential Bonferroni correction. CaEs that were significantly different expressed between a chemical treatment and the untreated control are shown in bold.

Clade	NCBI accession no.	*P*-value Effect of chemical treatment			
		Female		Male	
		IoA-00	IoA-02	IoA-00	IoA-02
J	HACA01002875.1	0.046*	0.027*	0.066	0.590
J	HACA01023258.1	0.354	0.857	0.698	0.069
E	HACA01030908.1	0.224	0.571	0.051	0.170
H	HACA01028197.1	0.589	0.839	0.354	0.169
H	HACA01016812.1	0.557	0.284	0.227	0.924
O	HACA01024270.1	0.927	0.815	0.124	0.077
O	HACA01010127.1	0.543	0.703	0.593	0.069
O	HACA01001173.1	0.133	0.505	0.242	0.083
O	HACA01028341.1	0.162	0.348	0.066	0.531
O	HACA01008519.1	0.156	0.326	0.884	0.244

*Significant at $P < 0.05$.

4.4.5 SNPs in CaE genes

Sequence variations in CaE genes that were predicted to be catalytically competent were identified by assessing RNAseq data available for individual male parasites of strains IoA-00 and IoA-02. Analyses revealed 15 SNP loci in five genes at which genotype frequencies differed significantly ($P < 0.05$) between the two strains (Table S4.8). Thirteen of these SNPs were missense mutations, i.e., encoded changes in the amino acid sequence, and ten of these mutations occurred in proximity of the protein's active site (Fig. S4.2). Three SNPs within CaE genes HACA01008519.1 (clade O; L374V and L375Q) and HACA01023258.1 (*ace1a*; F362Y) corresponding to missense mutations were fixed in all tested individuals of the multi-drug resistant strain IoA-02 while absent in drug-susceptible IoA-00 strain parasites. Mutations L374V and L375Q are located in proximity to the catalytic triad of the polypeptide encoded by HACA01008519.1. The mutation F362Y in AChE1a has previously been described and was demonstrated to be associated with resistance towards the organophosphate azamethiphos (Kaur et al., 2015b).

4.5 Discussion

This study presents the first genome and transcriptome-wide survey of the CaE family in *L. salmonis*, which led to the identification of 21 genes/pseudogenes coding for CaEs. The present study further examined potential roles of CaEs in the resistance of *L. salmonis* to salmon delousing agents by comparing transcript expression of selected CaEs between a drug-susceptible and a multi-resistant strain of the parasite. Abundance of two CaE transcripts (HACA01010127.1, clade O; HACA01002875.1, clade J, *ace1b*) was significantly increased in a multi-resistant strain compared to a drug susceptible reference strain of the parasite. Moreover, expression of HACA01002875.1 (*ace1b*) significantly increased ($P < 0.05$) in preadult-II females of both strains following exposure to sublethal concentrations of the macrocyclic lactone emamectin benzoate.

In the present study, the CaE gene family in *L. salmonis* was annotated using the phylogenetic classification scheme proposed by Oakeshott et al. (2005), which divides the family into 14 clades (A-N) within three classes. Additional taxonomically informative characters for much of the phylogeny are the catalytic competence and the cellular/subcellular localisation. The first dietary/detoxification class (clades A-C) contains catalytically competent enzymes with a wide range of cellular/subcellular localisations and comprises most CaEs involved in pesticide resistance in terrestrial arthropods. Members of the second hormone/semiochemical processing class (clades D-H) are catalytically competent, almost all secreted and, except for certain glutactins, not known to be membrane associated. In contrast, the third neuro/developmental

class (clades J-M) contains mostly catalytically incompetent proteins that are generally membrane associated (Oakeshott et al., 2005). Based on their phylogenetic similarity and much of their predicted catalytic competence and subcellular localisation, the *L. salmonis* CaE family can be partitioned into seven clades within two classes (Oakeshott et al., 2005).

None of the *L. salmonis* CaEs could clearly be assigned to the first class, known to possess detoxification functions (Oakeshott et al., 2005). In contrast, this class shows expansion in polyphagous or free-living ectoparasitic arthropods such as *D. melanogaster* (13 CaEs), *Tribolium castaneum* (26 CaEs), and *A. gambiae* (16 CaEs), which presumably need to detoxify a wide variety of xenobiotics during their lifecycle (Table S4.9). Salmon lice only ingest host products when feeding and are partially protected from environmental toxicants during host-attachment. Thus, the absence of detoxifying first class CaEs in *L. salmonis* may have arisen from a reduced exposure to environmental toxins (Claudianos et al., 2006; Teese et al., 2010). Similarly, the human body louse *Pediculus humanus,* which is an obligate blood feeder, and *A. mellifera*, which maintains a mutualistic symbiotic relationship with flowering plants, possess only three and nine CaEs in the detoxifying class, respectively (Claudianos et al., 2006; Lee et al., 2010) (Table S4.9). Supporting this hypothesis, *L. salmonis* has been shown to possess a markedly reduced number of genes encoding detoxifying ABC transporters (N=33) (Carmona-Antoñanzas et al., 2015) and CYPs (N=25) (Humble et al., 2019), compared to *D. melanogaster* (56 ABC transporters and 85 CYPs) or *T. castaneum* (73 ABC transporters and 131 CYPs) (Broehan et al., 2013; Dean et al., 2001; Oakeshott et al., 2010).

Three *L. salmonis* CaEs were assigned to clades H (glutactins) and E (secreted β-esterases) within the second hormone/pheromone and semiochemical processing class. Both *L. salmonis* glutactins have a conserved catalytic triad. Similarly, eight *A. aegypti* glutactins (N_{Total}=10) and one *D. melanogaster* glutactin (N_{Total}=4) are predicted to be catalytically active, although their substrates remain to be identified (Oakeshott et al., 2005; Strode et al., 2008). *L. salmonis* has one member (HACA01030908.1) in clade E, containing characterised secreted β-esterase from *D. melanogaster* (NP_001261749.1, Est-6; NP_788501.1, Est-7) (Chertemps et al., 2012; Dumancic et al., 1997; Meikle et al., 1990) and *A. mellifera* (NP_001011563.1) (Claudianos et al., 2006; Kamikouchi et al., 2004). Moreover, HACA01030908.1 encodes the *L. salmonis* CaE with the highest amino-acid similarity to validated β-esterases in *Popillia japonica* (AAX58713.1; Percent identity: 33.39%) (Ishida and Leal, 2008), *Antheraea polyphemus* (AAX58711.1; Percent identity: 30.95%) (Ishida and Leal, 2005; Vogt et al., 1985), and *Spodoptera littoralis (*ACV60237.1**,** Percent identity: 32.84%) (Durand et al., 2010). The above mentioned β-esterases have multiple functions, including metamorphic transition (NP_788501.1), reproductive functions (NP_001261749.1) (Meikle et al., 1990; Saad et al., 1994), degradation of plant

odorants (Durand et al., 2010), and pheromone signalling (Est-6; NP001011563.1; ACV60237.1; AAX58711.1; AAX58713.1) (Chertemps et al., 2012; Ishida and Leal, 2008; Ishida and Leal, 2005; Durand et al., 2010). Like other arthropods, the putative *L. salmonis* β-esterase is predicted to be soluble and secreted. However, the sequence lacks conserved catalytic triad residues, which would most likely render it catalytically inactive. Interestingly, molecular work on *D. virilis* and *D. buzzatii* has also recovered secreted β-esterases that lack an intact catalytic triad (reviewed in Robin et al., 2009). However, their function remains to be identified, complicating functional predictions for the putative β-esterase in *L. salmonis*.

Most *L. salmonis* CaEs belong to the third neuro/developmental class, which comprises five out of seven shared clades between *L. salmonis,* insects, and chelicerates (Grbic et al., 2011). CaE genes are known to evolve rapidly, and the neuro/developmental class is the most ancient group. Accordingly, this class harbours the only overlapping radiations of vertebrate, *C. elegans*, and arthropod CaEs (clades J, K, L) (Oakeshott et al., 2005, 1999). Except for AChE (J), all *L. salmonis* proteins within this class have an altered catalytic triad, indicating their hydrolytic inactivity. Based on the phylogenetic classification they are predicted to be involved in neurodevelopmental signalling and cell adhesion, i.e. neuroligins (clade L) have been implicated in synaptic growth, postsynaptic differentiation (Banovic et al., 2010; Sun et al., 2011), and sensory modulation (Biswas et al., 2010), neurotactins (clade M) have been characterised as being important for axon outgrowth, fasciculation, and guidance (Speicher et al., 1998), and gliotactins (clade K) have been shown to be responsible for septate junction formation (Genova and Fehon, 2003; Schulte et al., 2003) and the integrity of the transepithelial nerve-haemolymph permeability barrier (Auld et al., 1995).

The *L. salmonis* CaE family also comprises a new clade (clade O; five members), which could be found neither in the chelicerate *Tetranychus urticae* nor in insects (Table S4.9, S4.10). As explained above, CaEs are known to evolve rapidly. Thus, this CaE lineage may has evolved after the separation of the subphyla Crustacea and Hexapoda in the Cambrian (~525 million years ago) (Giribet and Edgecombe, 2019). Similarly, the CaE gene family of the chelicerate *T. urticae* comprises two clades that are absent in both crustaceans and insects and may have evolved after the separation of the chelicerata and mandibulata in the ediacaran (~550 million years ago) (Grbić et al., 2011) (Table S4.9).

The present study identified seven *L. salmonis* CaEs that contained an intact catalytic triad and three CaEs that grouped into clades of high bootstrap support with *D. melanogaster*, *A. mellifera*, or *L. salmonis* CaE sequences with a conserved catalytic triad. The transcript expression of these ten CaEs was characterised in two *L. salmonis* strains differing in drug susceptibility and following sublethal exposure to deltamethrin and emamectin benzoate. Five

out of ten tested CaEs showed significant sex/stage-biased transcript expression, with four transcripts being overexpressed by males. Sex-specific transcription of CaEs has previously been described in *L. salmonis* (Poley et al., 2016) and other arthropod species. For example, male-biased expression of CaE transcripts within the seminal fluid of *D. melanogaster* has been shown to affect physiological processes in females when transferred during mating (Richmond et al., 1980). Moreover, specific odorant degrading CaE transcripts overexpressed in males were found to play a role in refreshing the sensory system to continually respond to chemosensory signals such as female sex-pheromones (Chertemps et al., 2012). Sex-specific CaE transcript expression has also been linked to sexual dimorphisms in morphology or feeding pattern (Poley et al., 2016). In addition, CaEs can show developmental-specific expressions (Campbell et al., 2003). In the present study preadult-II female and adult male parasites were studied, so that the factors sex and stage are confounded, complicating the interpretation of CaE expression differences. Due to sex differences in *L. salmonis* size and development, the female preadult-II and male adult stages appear approximately at the same time in synchronised cohorts and are similar in size. Using these stages in this study ensured that all test animals experienced similar environmental conditions. Moreover, adult females of *L. salmonis* show significant within-stage growth and undergo cycles of oocyte production and vitellogenesis (Eichner et al., 2008), making this stage physiologically heterogeneous.

In the present study, expression of *ace1b* (HACA01002875.1, clade J) was significantly increased in multi-resistant IoA-02 salmon lice compared to drug susceptible IoA-00 parasites. The present study identified two *ace1* paralogues (*ace1a and ace1b)* in *L. salmonis,* confirming the findings of Kaur et al. (2015a). While AChE1a is predicted to be membrane bound, presumed to play the major role in cholinergic synaptic transmission, and the primary target for organophosphates, the physiological functions of AChE1b remain to be elucidated (Kaur et al., 2015a, 2015b). The present study predicts that AChE1b is soluble. In *A. mellifera* and *D. melanogaster*, soluble AChEs have been suggested to play a non-neuronal role of chemical defence as bioscavenger, thereby providing protection against pesticides before they arrive at their target sites (Kim et al., 2014, 2012; Lee et al., 2015). Accordingly, upregulation of *ace1b* in the multi-resistant strain IoA-02 compared to the drug susceptible strain IoA-00 may contribute to drug resistance by sequestration or hydrolysis. In the present study, exposure to emamectin benzoate caused significant upregulation of *ace1b* in females from strains IoA-00 and IoA-02. Soluble AChEs have also been shown to be overproduced in response to various stressors, including oxidative damage, psychological, physical, and chemical stressors (Birikh et al., 2002; García-Ayllón et al., 2012; Grisaru et al., 1999; Härtl et al., 2011; Lev-Lehman et al., 2000; Meshorer et al., 2002; Zimmerman and Soreq, 2006). Avermectins, which include emamectin benzoate, are chemical

stressors and have been shown to induce oxidative stress and DNA damage in crustaceans (Huang et al., 2019). As preadult-II female salmon lice have been found to be significantly more susceptible to emamectin benzoate than adult males (Carmona-Antoñanzas et al., 2016; Poley et al., 2015), the upregulation of *ace1b* in females may be a response to emamectin benzoate induced stress.
In the present study, expression of HACA01010127.1 (clade O) was significantly increased in multi-resistant IoA-02 salmon lice compared to drug susceptible IoA-00 parasites. Based on its phylogenetic classification and cytosolic localisation, HACA01010127.1 is most closely related to cytoplasmic/intracellular proteins with dietary and/or detoxification functions (Oakeshott et al., 2005). However, RACE sequencing of HACA01010127.1 revealed an altered catalytic triad, which would most likely render it catalytically inactive. To our knowledge, catalytically inactive proteins are not known to confer drug resistance.

In the present study, effects of drug exposures on CaE transcript expression were relatively moderate when determined at one time point after exposure. As gene induction can be a temporary event the experimental design may have failed to detect differential CaEs expression at earlier time points (Terriere, 1984). For example, in *M. domestica* time-dependent inductive expression patterns of CaEs have been observed within 12 to 72 h after permethrin challenge (Feng et al., 2018). Similarly, in *P. xylostella* pyrethroid exposure induced time-dependent alterations of carboxylesterase-6 mRNA expression levels within 3 to 48 h (Li et al., 2021). The design of exposure experiments in this report was aligned to recommendations for internationally standardised sea louse bioassays with deltamethrin and emamectin benzoate (Marín et al., 2018; Sevatdal and Horsberg, 2003; Westcott et al., 2008), allowing to compare results to those of other reports. In addition, in a previous study short emamectin benzoate exposures (1-3 h) resulted in very few transcripts being up- or down regulated (Carmichael et al., 2013). The experiment described in the present manuscript has been previously analysed with regards to drug exposure effects on CYP transcript expression, which was affected significantly by both deltamethrin and EBM in expression were found (Humble et al., 2019).

In addition to pesticide resistance mechanisms involving an enhanced expression of CaEs (Field and Foster, 2002; Wei et al., 2020), resistance may alternatively be conferred by point mutations of CaE genes altering enzyme specificity and/or activity. For example, single nucleotide substitutions in α-esterases leading to amino acid replacements in the catalytic centre have been shown to result in a loss of CaE activity and the acquisition of organophosphate hydrolase activity (Campbell et al., 1998; Claudianos et al., 1999; Newcomb et al., 1997). Furthermore, in *L. cuprina* mutations within the active site of CaEs have been shown to enhance the hydrolytic activity for several synthetic pyrethroids (Devonshire et al., 2007; Heidari et al., 2005). In the

present study, SNP analyses in CaE genes revealed that two genes contained non-synonymous mutations affecting amino acid residues near the active site gorge of the respective polypeptide, which were fixed in all sequenced individuals from the multi-drug resistant strain IoA-02 and absent in parasites from the drug-susceptible strain IoA-00. One of these mutations, F362Y in AChE1a, has previously been linked to organophosphate resistance in *L. salmonis* (Kaur et al., 2015b). The other two mutations occurred in HACA01008519.1 within clade O. More research is required to assess whether the mutation in HACA01008519.1 affect susceptibility of *L. salmonis* to salmon delousing agents.

The present study investigated the association of drug resistance with changes at the transcriptional level of CaEs. However, it is also conceivable that the enzymatic activity of CaEs have been altered by post-transcriptional and/or post-translational modifications. Following transcription, translation of CaE mRNAs can be regulated via modification of translation-initiation factors, regulatory protein complexes that recognise elements usually present in untranslated regions (UTRs) of the target mRNA or micro RNAs (miRNAs) that hybridise to mRNA sequences located in the 3′ UTR (Gebauer and Hentze, 2004). In addition, CaE enzyme activity can be altered by post-translational modifications such as amino acid changes, addition of macromolecules, or glycosylation, which have been implicated in protein stability and folding, targeting and recognition (Nalivaeva and Turner, 2001; Taylor and Feyereisen, 1996). For example, in organophosphate resistant *N. lugens* extensive differential post-translational glycosylation of CaE protein Nl-EST1 is believed to influence its stability, resulting in a non-linear correlation between Nl-EST1 mRNA levels and esterase activity (Small and Hemingway, 2000a, 2000b; Vontas et al., 2000). Another study suggested an association between organophosphate resistance in Australian cattle tick (*Rhipicephalus microplus)* strains and post-translational modifications producing a drug-insensitive AChE (Baxter and Barker, 2002, 1998).

Taken together, results from the present study suggest the potential involvement of *ace1b* (HACA01002875.1) in drug resistance in *L. salmonis*. However, it remains to be elucidated whether overexpression of *ace1b* is linked to deltamethrin, emamectin benzoate, and/or organophosphate resistance. No clear evidence was found for a role of other CaE genes in mediating resistance to emamectin benzoate or deltamethrin. Carmichael et al., 2013 found that expression of HACA01002103.1 (clade I; referred to as NP_001136104.1) was moderately enhanced in emamectin benzoate resistant salmon lice compared to a susceptible reference strain but, as shown in the present study, no significant differences in expression were apparent between susceptible and resistant salmon lice following emamectin benzoate exposure. Similarly, no evidence has been found for a role of CYP genes in mediating emamectin benzoate

resistance (Humble et al., 2019). Thus, the genes under selection of emamectin benzoate resistance in *L. salmonis* remain to be identified. For example, it has been suggested that emamectin benzoate resistance involves differential gene expression of P-gp (Heumann et al., 2012; Igboeli et al., 2012), GABA-gated chloride channels (Carmichael et al., 2013), and neuronal acetylcholine receptors (Carmichael et al., 2013; Poley et al., 2015). Similar to emamectin benzoate resistance, the present study provides no clear evidence for a role of CaE genes in mediating pyrethroid resistance, which is in line with studies by Poley et al. (2016) and Sevatdal et al. (2005).

Conclusion

The CaE gene family of *L. salmonis* is one of the smallest characterised in arthropods to date. It includes catalytically inactive genes predicted to be involved in neurodevelopmental function, as well as secreted catalytically competent genes. In addition, the *L. salmonis* CaE gene family contains a new clade, which is predicted to be largely catalytically competent and soluble. Results from the present study suggest an association of overexpression of *ace1b* (HACA01002875.1) with drug resistance in *L. salmonis*. No clear evidence was found for a role of other CaE genes in mediating resistance to emamectin benzoate or deltamethrin.

Chapter 5: General discussion

5.1 Summary of key findings

In the North Atlantic, resistance of *L. salmonis* towards the salmon delousing agent deltamethrin is widespread (Fjørtoft et al., 2020, 2019; Jensen et al., 2020; Sevatdal et al., 2005a). Previous studies provide evidence that deltamethrin resistance in *L. salmonis* is mainly inherited maternally and associated with mutations in the mitochondrial genome (Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015), suggesting novel still unresolved resistance mechanisms that are dissimilar to mechanisms known from terrestrial arthropods. These findings imply mitochondrial targets of deltamethrin toxicity in susceptible lice. In addition, *kdr*-type mutation I963V in voltage-gated sodium channel homologue LsNa$_v$1.3 has been suggested to contribute to deltamethrin resistance in *L. salmonis* (Carmona-Antoñanzas et al., 2019). In several terrestrial arthropods, metabolic detoxification by enzymes such as CaEs has also been shown to contribute to pyrethroid resistance (reviewed by Oakeshott et al., 2005). This PhD thesis investigated the relative contribution of Na$_v$1 target site mutations and mtDNA mutations to deltamethrin resistance in salmon lice. In addition, the potential involvement of the CaE gene family in the resistance phenotype was examined.

To disentangle potential roles of Na$_v$1 target-site mutations and mtDNA mutations in deltamethrin resistance in *L. salmonis*, the studies presented in this thesis investigated salmon lice samples from diverse origin and applied a variety of genetic and toxicological analyses. *L. salmonis* were obtained from a range of Scottish farm sites, rated as deltamethrin resistant or susceptible in bioassays, and genotyped at LsNa$_v$1.3 SNP I936V and selected mitochondrial SNPs. Samples tested further included *L. salmonis* from deltamethrin resistant and susceptible laboratory-maintained strains, lice derived from crosses of strains differing in deltamethrin susceptibility, and lice collected from wild hosts. Genetic tests were complemented by bioassays with the non-ester pyrethroid etofenprox, a compound that has been used to detect target-site resistance based on Na$_v$1 mutations. LsNa$_v$1.3 SNP I936V was not associated with deltamethrin resistance in *L. salmonis* from diverse origin and a deltamethrin resistant *L. salmonis* strain did not show cross-resistance to etofenprox, suggesting that Na$_v$1 target-site mutations do not play a major role as determinants of deltamethrin resistance in salmon lice.

A set of mtDNA mutations that has previously been linked to deltamethrin resistance in laboratory-maintained strains of *L. salmonis* was investigated regarding its associated with deltamethrin resistance in lice collected from the field. Analysis of results revealed 11 mtDNA haplotypes of which three were associated with deltamethrin resistance, named 2, 3, and 4, contrasting findings by Carmona-Antoñanzas et al. (2017) who reported mtDNA sequences consistent with haplotype 2 for all deltamethrin resistant isolates analysed in their study.

Phylogenetic analyses of haplotypes suggested that deltamethrin resistance evolved at least two times independently. Resistant haplotype 3 was most distinct from the previously characterised resistant haplotype 2 (Carmona-Antoñanzas et al., 2017) and selected for further studies, which involved crossing experiments to assess the mode of inheritance of deltamethrin resistance conferred by this haplotype and toxicity experiments to investigate its susceptibility to ATP depletion during deltamethrin exposure. Despite haplotype 3 being very different in sequence, both lice with haplotypes 2 and 3 were highly deltamethrin resistant and transmitted their resistance to the next generation through maternal inheritance. This indicated that deltamethrin resistance in these lice is conferred by the same mechanism associated with the same mtDNA mutations. Comparison of mtDNA haplotypes suggested the association of deltamethrin resistance with SNP T8600C, corresponding to Leu107Ser in COX1. MtDNA mutation T8600C was also the only non-synonymous mutation common to all deltamethrin resistant lice but lacking in all drug-susceptible parasites.

To investigated potential roles of CaEs in deltamethrin resistance in *L. salmonis*, sequences encoding CaEs were identified in the *L. salmonis* genome and annotated, and CaE sequences predicted to be catalytically competent were studied regarding their transcript and SNP expression in resistant and susceptible lice. The study revealed that the CaE gene family of *L. salmonis* is one of the smallest characterised in arthropods to date. It includes catalytically inactive proteins predicted to be involved in neurodevelopmental function, secreted catalytically competent enzymes, and a new clade that is predicted to be largely catalytically competent. Analysis of transcript expression and SNPs suggested that CaE are not major determinants of pesticide resistance but could have relevance as factors contributing to it.

5.2 Target-sites of deltamethrin toxicity

In terrestrial arthropods, deltamethrin toxicity is based on drug binding and blockage of Na_v1, resulting in the disruption of neurotransmission (Dong et al., 2014). However, due to their lipophilic nature pyrethroids can pass and interact with biological membranes, making a variety of other membrane proteins and structures candidate targets for pyrethroid action (Guven et al., 2018). In the crustacean species *L. salmonis* the mechanisms of deltamethrin resistance are largely unknown. Previous studies suggest that other mechanisms than classical Na_v1 target-site mutations are contributing to the deltamethrin resistance phenotype in this species (Bakke et al., 2018; Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015). For this reason, the following sections briefly summarises the current state of knowledge on potential target-sites of pyrethroid toxicity.

In addition to their effect on Na_v1, type II pyrethroids can modify the gating kinetics of voltage-gated calcium channels and have been suggested to block voltage-gated chloride channels (Breckenridge et al., 2009; Symington and Clark, 2005). Moreover, they can bind and inhibit GABA-gated chloride channels (Costa, 2015; Kumar Singh et al., 2012), and have been shown to affect nicotinic acetylcholine receptors (Abbassy et al., 1983a, 1983b, 1982; Oortgiesen et al., 1989) and glutamate receptors (Frey and Narahashi, 1990; Staatz et al., 1982). Deltamethrin was also found to decrease the AChE activity in brain of rats (Khan et al., 2018; Saoudi et al., 2017), which has been attributed to a reduction of the acetylcholine binding space (Khan et al., 2018, 2013).

Both type I and type II pyrethroids have been shown to induce the generation of ROS in both the cytosol and the mitochondria (Klimek, 1990; Truong et al., 2006; Vontas et al., 2001), which can trigger a cascade of reactions that induces lipid peroxidation and damage of macromolecules (Chirico et al., 1993; Fang et al., 2002; Maiti et al., 1995). In addition, they can affect mitochondrial membrane structures and dynamics (Braguini et al., 2004; Zhang et al., 2007) and induce inhibition of mitochondrial respiratory complexes (Braguini et al., 2004; Gassner et al., 1997), which can disrupt the mitochondrial oxidative phosphorylation.

Pyrethroids have also been shown to induce apoptosis in different mammalian cell types by mechanisms involving intrinsic (mitochondrial) (Kumar et al., 2016) and extrinsic (death receptor) pathways (Arslan et al., 2017), as well as the ER stress pathway (Hossain and Richardson, 2011).

Taken together, deltamethrin toxicity in salmon lice may involve a variety of molecular targets against which different resistance mechanisms may have evolved. To obtain insights into underlying molecular mechanisms, this PhD thesis focused on investigating potential roles of Na_v1 target-site mutations that prevent binding and blocking of deltamethrin to Na_v1, as well mtDNA mutations that may provide protection against deltamethrin induced disruption of mitochondrial oxidative phosphorylation and intrinsic mitochondrial apoptosis.

5.3 Mechanisms of deltamethrin resistance in *L. salmonis* and implications for mode of action

5.3.1 Na_v1 target-site mutations

While pyrethroid toxicity can involve a variety of molecular sites, Na_v1 is considered the main target-site in terrestrial arthropods. Specific non-synonymous point mutations within Na_v1 (so called *kdr* mutations) have been shown to diminish the channel's susceptibility to pyrethroids by altering its gating kinetics and/or reducing its binding affinity for pyrethroids (Oliveira et al., 2013; Tan et al., 2005; Vais et al., 2003, 2000).

To date, more than 50 *kdr* mutations/combinations of *kdr* mutations have been identified and many of them have been functionally confirmed to be associated with pyrethroid resistance by recombinant expression in *Xenopus* oocytes (Dong et al., 2014). Most *kdr* mutations map to two pyrethroid-binding sites within Na_v1 (Du et al., 2013; O'Reilly et al., 2006) and several *kdr* mutations have evolved independently in different arthropod species (Davies et al., 2007b; Eleftherianos et al., 2008; Guerrero et al., 1997; Haddi et al., 2012; Toda and Morishita, 2009; Williamson et al., 1996).

In *L. salmonis*, three homologues of Na_v1 have been identified, which are named $LsNa_v1.1$, $LsNa_v1.2$ and $LsNa_v1.3$ (Carmona-Antoñanzas et al., 2019). Characterisation of the three Na_v1 homologues led to the identification of one putative *kdr* mutation in $LsNa_v1.3$ (Carmona-Antoñanzas et al., 2019), which causes an amino acid change (I936V, numbering according to *M. domestica* Na_v1) in the predicted pyrethroid binding site of the channel and is homologous to a previously described *kdr* mutation in pyrethroid-resistant isolates of the phytophagous moth *H. zea* (Hopkins and Pietrantonio, 2010; O'Reilly et al., 2006; Usherwood et al., 2007).

The study presented in chapter I of this PhD thesis used pharmacological and genetic approaches to investigate whether $LsNa_v1.3$ I936V is involved in deltamethrin resistance in salmon lice. Following a pharmacological approach, experiments were conducted with the non-ester pyrethroid etofenprox, whose chemical structure contains an ether bridge replacing the central ester group present in conventional pyrethroids (Nishimura et al., 1986). Ligand docking studies on a Na_v1 model revealed similar binding positions of fenvalerate, the base molecule for etofenprox, and deltamethrin (O'Reilly et al., 2006; Usherwood et al., 2007), and several *kdr* mutations have been reported to confer cross-resistance between etofenprox, fenvalerate, and deltamethrin (Beddie et al., 1996; Farnham et al., 1987; Pedersen, 1986; Soderlund, 2008). Thus, target-site mutations in *L. salmonis* Na_v1 homologues would be expected to confer cross-resistance between etofenprox and deltamethrin. However, in the present PhD study a deltamethrin resistant *L. salmonis* strain did not show cross-resistance to etofenprox, which suggests that Na_v1 target-site mutations do not play a major role as determinants of deltamethrin resistance in salmon lice. Pharmacological findings could be confirmed by genetic analyses. Based on acute toxicity tests carried out with adult/preadult parasites, mutation $LsNa_v1.3$ I936V was not associated with deltamethrin resistance in *L. salmonis* from farm sites and in F2 lice derived from a deltamethrin resistant male and a drug-susceptible female (Tschesche et al., 2020). Taken together, experiments with both pharmacological and genetic approaches did not provide evidence for major roles of $LsNa_v1.3$ I936V in deltamethrin resistance of *L. salmonis*.

Nonetheless, protective roles of the mutation cannot be excluded with other exposure scenarios or for other life stages. The three *L. salmonis* Na_v1 paralogues have been shown to be differentially expressed in different life stages/sexes and following drug exposure (Carmona-Antoñanzas et al., 2018). Similarly, mammalian Na_v paralogues differ in channel gating kinetics, ontogeny, and tissue expression profiles, with certain isoform primarily involved in the nervous response and others responsible for the excitation of skeletal or cardiac myocytes (Yu and Catterall, 2003). In *L. salmonis*, cellular localisation and functional roles of the Na_v1 homologues are still unknown, but their presence may indicate subfunctionalisation such as expression in different types of neurones with heterogeneous functions in different life stages. In this case, toxic effects of deltamethrin on channel homologues may not be visible in acute bioassays with adult/preadult salmon lice. For example, deltamethrin may kill lice in a longer timeframe by affecting nerves involved in feeding mechanisms in certain life stages, or deltamethrin compromises lice attachment by impairing mechanisms for clinging onto fish. Accordingly, $LsNa_v1.3$ mutation I936V may confer protection against toxic effects that could not be detected by the experimental design chosen in the present study. However, as the cellular localisation and functional role of $LsNa_v1.3$ remain to be identified, the channel may also have no or only secondary relevance as a molecular target-site for acute toxic effects of deltamethrin in adult and preadult *L. salmonis*. Deltamethrin toxicity in these parasite stages could potentially be mediated through other molecular targets such as Na_v1 homologues $LsNa_v1.1$ and/or $LsNa_v1.2$, or hypothetical mitochondrial targets (Carmona-Antoñanzas et al., 2017). In contrast to mitochondrial targets (which are discussed in the next section), $LsNa_v1.1$ and $LsNa_v1.2$ may be less likely to be involved in deltamethrin toxicity in salmon lice because despite the extensive usage of deltamethrin during the last two decades no *kdr*-type mutations were found in these homologues in deltamethrin resistant lice from Scotland and Norway (Carmona-Antoñanzas et al., 2019; Helgesen, 2015).

A previous study by Jensen et al. (2017) supports the hypothesis that deltamethrin toxicity in adult and preadult *L. salmonis* is not primarily based on the blockage of Na_v1. The authors found that the median survival time of susceptible lice treated with the recommended treatment dosage of deltamethrin was 16.8 h, while the corresponding median survival time for the neurotoxic compound azamethiphos was considerably shorter, 16 min. As neurotoxic compounds have been shown to induce rapid knock-down effects (within 60 min in *M. domestica*) (Scott and Georghiou, 1984), the authors suggested that deltamethrin toxicity in *L. salmonis* does not primarily involve Na_v1.

5.3.2 MtDNA mutations

Besides target-site mutations in Na_v1, previous studies provide evidence that deltamethrin resistance in *L. salmonis* is mainly inherited maternally and associated with mutations in the mtDNA (Bakke et al., 2018; Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015).

To disentangle roles of nuclear and mitochondrial mutations in deltamethrin resistance in salmon lice, chapter I of this PhD thesis tested previously described mtDNA SNPs (Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015) versus $LsNa_v1.3$ SNP I936V as genetic markers for resistance in the field. In contrast to $LsNa_v1.3$ I936V, genotypes at the tested mtDNA loci were significantly associated with deltamethrin resistance, suggesting mitochondrial determinants for deltamethrin resistance in *L. salmonis* (Carmona-Antoñanzas et al., 2017).

Chapter II of this PhD thesis investigated the relationship between mitochondrial SNPs and deltamethrin resistance further, aiming to obtain deeper insights into the involvement of mitochondrial SNPs in the resistance mechanism and to pinpoint the most accurate deltamethrin resistance marker. Identifying mtDNA SNPs that are casually linked to deltamethrin resistance is challenging, as they must be differentiated from hitchhiking SNPs that are associated with resistance without being involved in its mechanism. Hitchhiking SNPs can accumulate in the mtDNA because the inheritance of mtDNA is linear and lacks recombination through meiosis (Scheffler, 2001). In addition, the mtDNA of invertebrates has a high mutation rate with substitutions being integrated nine times faster than in nuclear genes (Lynch et al., 2006; Tjensvoll et al., 2006). Accordingly, only SNPs that are present in all resistant lice but lacking in all susceptible individuals come into question as SNPs causally linked to deltamethrin resistance.

To pinpoint mtDNA SNPs that are potentially causally linked to deltamethrin resistance, *L. salmonis* obtained from farm sites were subjected to deltamethrin bioassays and genotyping at 18 previously identified deltamethrin resistance-associated mitochondrial SNP loci (Carmona-Antoñanzas et al., 2017) to identify mitochondrial haplotypes and assess their association with deltamethrin resistance. Genotyping further included historic samples obtained from wild hosts (2010) and resistant laboratory strain isolates (2013). The resistant haplotype that differed maximally from the resistant haplotype characterised by Carmona-Antoñanzas et al. (2017) was selected for further studies, which involved assessing the mode of inheritance of deltamethrin resistance conferred by the haplotype. Comparing the mtDNA sequence between well-characterised resistant and susceptible *L. salmonis* strains allowed narrowing down mtDNA SNPs that come into question as SNPs causally linked to deltamethrin resistance.

Genotyping of *L. salmonis* from farm sites, laboratory strains, and wild hosts revealed three mtDNA haplotypes associated with deltamethrin resistance, named 2, 3, and 4, of which haplotype 2 was found in 75% of all resistant lice. This finding contrast results by

Carmona-Antoñanzas et al. (2017), who reported mtDNA sequences consistent with haplotype 2 for all deltamethrin resistant isolates analysed in their study. While resistance-associated haplotypes 2 and 4 shared 17 out of 18 mtDNA SNPs, resistance-associated haplotype 3 differed maximally in sequence. To investigate the relationship of haplotypes 2 and 3 to deltamethrin resistance, the present study performed comparative experiments with strain IoA-02 containing haplotype 2 and strain IoA-10 containing haplotype 3. Despite haplotypes 2 and 3 being very different in sequence, both IoA-02 (haplotype 2) and IoA-10 (haplotype 3) lice were highly deltamethrin resistant (~100-fold; $P = 0.845$) and reciprocal crosses of strains IoA-02 (Carmona-Antoñanzas et al., 2017) and IoA-10 (present study) with drug-susceptible lice revealed that both strains transmit their resistance to the next generation through maternal inheritance. These results provide further support for mtDNA being the basis for maternal effects (Bakke et al., 2018; Carmona-Antoñanzas et al., 2017). Furthermore, the high level of deltamethrin resistance of IoA-02 lice containing haplotypes 2 and IoA-10 lice containing haplotype 3 suggests that deltamethrin resistance in both strains is conferred by the same mechanism, which is associated with the same mtDNA mutation(s). The only mtDNA SNP shared by haplotypes 2 and 3 is T8600C, which corresponds to an amino acid change of Leu107Ser in COX1 of electron transport chain (ETC) complex IV. The mutation is also present in the resistance-associated haplotype 4 but is lacking in all susceptibility-associated haplotypes. When comparing mtDNA sequences, T8600C is also the only non-synonymous mutation differentiating between deltamethrin resistant and susceptible individuals. Moreover, the mutation has previously been linked to deltamethrin resistance in two independent studies on Norwegian *L. salmonis* strains (Bakke et al., 2018; Nilsen and Espedal, 2015). These findings provide evidence that deltamethrin resistance in *L. salmonis* is associated with mtDNA SNP T8600C (Leu107Ser in COX1). This suggests a deltamethrin resistance mechanism unique for *L. salmonis*. To my knowledge, mtDNA mutations have only been described as determinants for resistance against specific acaricide. For example, in the spider mite *T. urticae* bifenazate resistance was found to be highly correlated with mutations in cytB of ETC complex III (Leuween et al., 2008).

It must be noted that sequencing analyses of *L. salmonis* from diverse origin further revealed eight additional SNPs in non-coding regions (NCRs) of the mtDNA that were common to all resistant lice and lacking in all susceptible lice. However, NCRs are the most variable mtDNA sequences, which may explain the high number of SNPs found within these regions (Nicholls and Minczuk, 2014). Seven SNPs were found in the mitochondrial control region, also known as displacement loop, which is not known to confer drug resistance (Clayton, 1991, 1982). The other SNP A10178G was found within a mitochondrial ribosomal RNA (rRNA) gene and has previously been described by Bakke et al. (2018). Mutation within the mitochondrial rRNA may

lead to ribosome dysfunction and may result in respiratory chain defects (Smith et al., 2014; Sylvester et al., 2004). However, to my knowledge, there are no reports on mitochondrial rRNA mutations associated with drug resistance. Thus, most likely the eight SNPs identified in NCRs are only non-causatively liked to the resistance phenotype.

This PhD study also found that deltamethrin exposure caused behavioural toxicity and whole-body ATP depletion in drug susceptible parasites but not deltamethrin resistant IoA-10 (haplotype 3) and IoA-02 (haplotype 2) lice, which is in line with an earlier experiment performed by Carmona-Antoñanzas et al. (2017). These results suggest that depletion of ATP levels in deltamethrin susceptible lice is related to toxic effects of deltamethrin on the mitochondria and mtDNA SNPs in resistant lice, most likely T8600C, may have a protective effect.

What do these findings implicate for the mechanism of deltamethrin resistance and, by inference, the mechanism of deltamethrin toxicity in *L. salmonis*?

Toxic effects of pyrethroids on mitochondrial functions have been described in several studies with terrestrial arthropods and mammals. Due to their lipophilic nature, pyrethroids can pass and interact with biological membranes, making mitochondrial membranes and membrane proteins candidate targets for toxic action (Guven et al., 2018). For example, pyrethroids have been shown to affect mitochondrial membrane structures and dynamics, which can impair oxidative phosphorylation (Braguini et al., 2004; Zhang et al., 2007). Moreover, several studies reported pyrethroid induced inhibition of mitochondrial respiratory complexes, which can affect oxidative phosphorylation too. In isolated rat liver mitochondria, permethrin and cyhalothrin caused inhibition of respiratory complex I (Gassner et al., 1997), and deltamethrin was predicted to have a major inhibition site between respiratory complexes II and III (Braguini et al., 2004). Mitochondrial oxidative phosphorylation can also be impaired by intracellular Ca^{2+} accumulation (Bauer and Murphy, 2020; Paschen, 2000), which can result from interactions of pyrethroids with Na_v1 and consequent Ca^{2+} influx (Hossain and Richardson, 2011) and direct effects of pyrethroids on voltage gated Ca^{2+} channels (reviewed by Clark and Symington, 2011). Within the mitochondria, pyrethroids induced disruption of mitochondrial membrane integrity and inhibition of respiratory complexes as well as intracellular Ca^{2+} accumulation can cause the generation of ROS (Brookes et al., 2004; Ildiko Sipos et al., 2003; Truong et al., 2006). ROS can trigger a cascade of reactions that induces lipid peroxidation and damage of macromolecules (Chirico et al., 1993; Fang et al., 2002; Maiti et al., 1995), which can also cause disruption of oxidative phosphorylation (Fariss et al., 2005). In addition to their role in mitochondrial energy metabolism, pyrethroids have been shown to induce intrinsic mitochondrial apoptosis (Ko et al., 2016; Kumar et al., 2016). This pathway involves mitochondrial outer membrane permeabilization (MOMP), release of cytC into the cytosol, binding of cytC to apoptotic

peptidase activating factor 1, activation of caspases, and ultimately DNA fragmentation (Bock and Tait, 2020). MOMP is considered a point-of-no-return in apoptosis execution (Kroemer, 2003). Among others, it can be triggered by pyrethroid induced oxidative stress and Ca^{2+} accumulation (Buttke and Sandstrom, 1994; Hajnóczky et al., 2003; Lenaz, 1998). The mitochondrial apoptotic pathway can also be induced by low levels of ATP that lead to disruption of the mitochondrial transmembrane potential. In addition, increased mitochondrial permeability and apoptosis has been linked to enhanced expression of pro-apoptotic p53 and Bax and decreased expression of anti-apoptotic Bcl-2 following deltamethrin exposure (Wu et al., 2000; Wu and Liu, 2000a, 2000b).

Based on these findings, an obvious explanation for deltamethrin toxicity in *L. salmonis* would be that deltamethrin is binding to a mitochondrial respiratory complex, which is leading to the disruption of the ETC and thus the mitochondrial ATP production, the latter of which was observed in the present study and by Carmona-Antoñanzas et al. (2017). Resistance may be conferred by mitochondrial SNP(s) that changes the amino acid sequences of the complex and impair binding of deltamethrin. Similarly, in *T. urticae* the acaricide fluacrypyrim has been shown to inhibit the mitochondrial oxidative phosphorylation by binding to electron transport complex III and resistance has been shown to be conferred by mutations in the compound's binding site (Van Nieuwenhuyse et al., 2009). The most likely candidate mutation for conferring deltamethrin resistance in *L. salmonis* is T8600C leading to Leu107Ser in COX1 of respiratory complex IV. As explained above, T8600C was only non-synonymous mutation differentiating between deltamethrin resistant and susceptible individuals when comparing mtDNA sequences. Moreover, the mutation has previously been linked to deltamethrin resistance in two independent studies on Norwegian *L. salmonis* strains (Bakke et al., 2018; Nilsen and Espedal, 2015).

Respiratory complex IV is an integral protein in the inner mitochondrial membrane. It is composed of 14 protein subunits of which three are synthesised in the mitochondria, i.e., COX1, COX2, and COX3 (Balsa et al., 2012). The complex catalyses the reduction of O_2 to water at the heme a_3-Cu_B binuclear centre within COX1, by means of electrons from cytC and protons extracted from the matrix site, in a reaction coupled with proton pumping. Interestingly, Leu107Ser is located on the surface of the COX1 protein structure, making it an available target for binding with deltamethrin (Bakke et al., 2018). However, this hypothesis needs further validation. Firstly, *in silico* docking of deltamethrin to respiratory complex IV has not yet been conclusive (Bakke et al., 2018). Secondly, Leu107Ser is located at a distance to the heme a_3-Cu_B binuclear centre (positioned within amino acids 234 to 289), which is the target-site of most inhibitors of complex IV, such as cyanide, nitric oxide, and carbon monoxide.

Alternatively, deltamethrin may cause toxicity by impairing the mitochondrial ATP production through disruption of mitochondrial membranes, intracellular Ca^{2+} accumulation, or induction of oxidative stress (Chinopoulos et al., 2000; Clark and Symington, 2011; Hossain and Richardson, 2011). If deltamethrin toxicity involves an impairment of the mitochondrial ATP production through these scenarios, mtDNA mutation(s) may have functional effects on the efficiency of electron transfer or proton translocation, counteracting ATP deficits. In a pervious study (Carmona-Antoñanzas et al., 2017), deltamethrin susceptible (IoA-00) and resistant (IoA-02) control animals showed similar whole-body ATP levels. Thus, mtDNA mutations does not seem to adjust the efficiency of electron transfer/proton translocation in a way that changes the baseline ATP levels of their carrier. However, rates to replenish ATP levels after disturbance of equilibrium may be increased in parasites displaying these mutations.

Deltamethrin toxicity in *L. salmonis* may also involve activation of apoptotic pathways. Accordingly, deltamethrin exposure increased apoptosis in mitochondria-rich skeletal muscle, subcuticular tissue, and central ganglion cells in salmon lice of a drug susceptible strain, but not or to a lesser degree in a deltamethrin resistant strain (Bakke et al., 2018). Deltamethrin may trigger the mitochondrial apoptotic pathway in these tissues through generation of ROS or low levels of ATP, the latter of which was observed in the present study. MtDNA mutations may provide protection against deltamethrin induced intrinsic mitochondrial apoptosis in two different ways, which are explained above. MtDNA mutations may impair binding of deltamethrin to a mitochondrial respiratory complex, which prevents disruption of the mitochondrial ATP production and generation of ROS, or they may have functional effects on the efficiency of electron transfer or proton translocation, counteracting ATP deficits.

5.3.3 Metabolic detoxification

While results from this PhD study strongly support the view that mitochondrial mutations play the predominant role among genetic factors causing deltamethrin resistance in *L. salmonis*, findings also suggest the contribution of additional nuclear determinants. Firstly, susceptible individuals derived from a deltamethrin resistant IoA-10 sire and a susceptible IoA-00 dam (EC_{50} 0.55 µg l^{-1}) were significantly less sensitive to deltamethrin than fully pedigreed IoA-00 lice (EC_{50} 0.25 µg l^{-1}) (see chapter II). Secondly, 20% of the F2 parasites descending from a deltamethrin susceptible IoA-00 dam and a resistant IoA-02 sire was deltamethrin resistant (Carmona-Antoñanzas et al., 2017). Thirdly, 43% of field-collected lice with a deltamethrin susceptibility-associated mitochondrial haplotype were classified as deltamethrin resistant in bioassays (chapter II). And finally, moderate levels of deltamethrin resistance had

already been reported in the early 2000s (Sevatdal et al., 2005a), almost a decade before a resistance-associated mitochondrial haplotype first emerged (Fjørtoft et al., 2020).

Besides target-site mutations in Na_v1, pyrethroid resistance in terrestrial arthropods can involve enhanced detoxification by enzymes such as CYPs, GSTs, and CaEs, which are encoded by nuclear genes (Panini et al., 2016).

Before this PhD project, little was known about the CaE family in *L. salmonis* and its roles in drug resistance. The study presented in chapter III of this thesis identified and annotated *L. salmonis* CaE homologues, and CaE sequences predicted to be catalytically competent were selected for further analyses as they have the potential to mediate pesticide resistance. Transcript expression of potentially catalytically competent CaEs was studied using quantitative reverse-transcription PCR in drug susceptible and multi-resistant *L. salmonis*, and effects of deltamethrin exposure were assessed. In addition, SNPs in CaE sequences were identified by comparing available RNA-seq data between a multi-resistant and a drug-susceptible laboratory-maintained strain.

The present study identified 21 CaEs genes/pseudogenes in *L. salmonis*. Based on the phylogenetic classification scheme by Oakeshott et al. (2005), which divides the CaE family into 14 clades within three classes, none of the *L. salmonis* CaEs could clearly be assigned to the first class known to possess detoxification functions (Oakeshott et al., 2005, 2010). Similarly, the chelicerate *T. urticae* has no members within this class (Grbić et al., 2011). CaEs are known to evolve rapidly (Oakeshott et al., 2005). Thus, this class may be an insect specific radiation that evolved after the divergence from the crustacean lineage in the Cambrian (~525 million years ago) (Giribet and Edgecombe, 2019). Accordingly, other CaEs may take up detoxification functions in *L. salmonis*.

However, pesticide resistance can only be mediated by catalytically active CaEs, which possess a catalytic triad with a nucleophilic residue (Ser, Cys, or Asp), an acidic residue (Glu or Asp), and a histidine residue (Myers et al., 1988). Similar to other species, the *L. salmonis* CaEs family is functionally very diverse (Oakeshott et al., 2005; Tschesche et al., 2021). It comprises secreted enzymes within the second hormone/semiochemical processing class, as well as several catalytically incompetent proteins involved in neurodevelopmental signalling and cell adhesion within the third class. In addition, the *L. salmonis* CaE family contains a new clade (named clade O), which could be found neither in *T. urticae* nor in insects (Claudianos et al., 2006; Grbić et al., 2011; Lee et al., 2010; Oakeshott et al., 2010; Yu et al., 2009) and thus, may have evolved after the separation of the crustacean lineage (Giribet and Edgecombe, 2019).

In total, ten *L. salmonis* CaEs were identified as being potentially catalytically competent and thus, have the potential to mediate pesticide resistance. Potentially catalytically active

L. salmonis CaEs include 2 glutactins and 1 secreted β esterase (class 2), 2 AChEs (*ace1a and ace1b, class 3)*, and 5 CaEs within the new clade O. Catalytically active CaEs can mediate pesticide resistance by hydrolysis or sequestration (Hemingway, 2000). During hydrolysis ester pesticides such as pyrethroids are cleaved into their corresponding acid and alcohol metabolites, which usually show low toxicity and are excreted readily. In contrast, sequestration can involve both ester and non-ester pesticides, leading to impaired interactions with their toxicological target-sites (Hemingway, 2000). When assessing potential roles of CaEs in detoxifying pyrethroids, it must therefore be considered that CaE related mechanisms are not specific to one pesticide class. In addition, interpretation of results of the present study is complicated by the fact that the main model strain is multi-drug resistant, with resistance levels based on acute bioassays being 143-fold for deltamethrin, 4.3 to 7.3-fold for EMB, and 23-fold for azamethiphos (Carmona-Antoñanzas et al., 2017, 2016; Humble et al., 2019).

In terrestrial arthropods, CaEs can confer pesticide resistance by different molecular mechanisms (reviewed by Hemingway, 2000). Firstly, gene amplification (Field and Devonshire, 1998; Rooker et al., 1996) or selection of overexpressed alleles can lead to enhanced CaE expression (Wei et al., 2020; Zhu and Luttrell, 2015). As these mechanisms are linked to changes in transcript expression their presence in the drug resistant salmon louse isolates would have been detected by the qPCR analyses performed in the present study. Results showed that expression of *ace1b* was significantly increased in multi-resistant salmon lice compared to drug susceptible parasites (Tschesche et al., 2021). While AChE1a is predicted to be membrane bound and presumed to play the major role in cholinergic synaptic transmission, AChE1b is predicted to be soluble (Tschesche et al. 2021) but its physiological functions remain to be elucidated (Kaur et al., 2015a, 2015b). In *A. mellifera* and *D. melanogaster*, soluble AChEs have been suggested to play a non-neuronal role in chemical defence as bioscavenger, thereby providing protection against pesticides before they arrive at their target sites (Kim et al., 2014, 2012; Lee et al., 2015). Accordingly, upregulation of *ace1b* in the multi-resistant strain compared to the drug susceptible strain may contribute to drug resistance by sequestration or hydrolysis. In this context, it remains to be elucidated whether overexpression of *ace1b* is linked to deltamethrin, EMB, and/or organophosphate resistance. However, it is also possible that upregulation of soluble *ace1b* was not linked to any of the resistance mechanisms, but to an unrelated trait. For example, soluble AChE is also believed to be involved in reducing the concentration of free acetylcholine, which increases during stress induced hyperexcitation (Grisaru et al., 1999; Niar and Hunter, 2004).

No clear evidence was found for a role of other CaE genes in mediating resistance to deltamethrin.

Besides pesticide resistance mechanisms involving enhanced expression of CaEs (Field and Foster, 2002; Wei et al., 2020), resistance can also be conferred by point mutations in CaE genes that alter the enzyme's specificity and/or activity (Claudianos et al., 1999; Devonshire et al., 2007; Heidari et al., 2005; Newcomb et al., 1997). In the present PhD study, SNP analyses revealed two CaEs genes with non-synonymous mutations near the active site gorge, which were present in all sequenced individuals from the multi-drug resistant strain and lacking in all parasites from the drug-susceptible strain. One of these mutations, F362Y in AChE1a, has previously been described and linked to organophosphate resistance in *L. salmonis* (Kaur et al., 2015b). The other two mutations were identified here for the first time and occurred in a CaE gene within clade O. However, more research is required to assess whether the mutations affect the susceptibility of *L. salmonis* towards the salmon delousing agent deltamethrin.

This PhD thesis investigated the association of drug resistance with changes at the transcriptional level of CaEs. However, the enzymatic activity of CaEs can also be altered by post-transcriptional and/or post-translational modifications. Following transcription, translation of CaE mRNAs can be regulated via modification of translation-initiation factors, regulatory protein complexes that recognise elements usually present in UTRs of the target mRNA or miRNAs that hybridise to mRNA sequences located in the 3′ UTR (Gebauer and Hentze, 2004). In addition, CaE enzyme activity can be altered by post-translational modifications such as amino acid changes, addition of macromolecules, or glycosylation, which have been implicated in protein stability and folding, targeting and recognition (Nalivaeva and Turner, 2001; Taylor and Feyereisen, 1996). For example, in organophosphate resistant *N. lugens* extensive differential post-translational glycosylation of CaE protein Nl-EST1 is believed to influence its stability, resulting in a non-linear correlation between Nl-EST1 mRNA levels and esterase activity (Small and Hemingway, 2000a, 2000b; Vontas et al., 2000).

Taken together, the study presented in chapter III of this PhD thesis did not find clear evidence for roles of CaE genes in mediating deltamethrin resistance in salmon lice.

Similarly, Humble et al. (2019) found no evidence for a role of CYP genes in the decreased susceptibility of the multi-resistant parasite strain studied in the present experiment. However, in both susceptible and resistant lice there will be some metabolism of deltamethrin, and CaEs and CYPs may well be involved. Besides CaEs and CYPs, serine proteases (Gong et al., 2005; Yang et al., 2008b), GSTs (Grant and Matsumura, 1989; Reidy et al., 1990), and ABC transporters (reviewed by Dermauw and Van Leeuwen, 2014) have been shown to be involved in pyrethroid resistance in terrestrial arthropods. In *L. salmonis*, these enzymes and transporters have been studied elsewhere (Carmona-Antoñanzas et al., 2015; Heumann et al., 2014, 2012; Igboeli et al.,

2012; Poley et al., 2016), however, their potential roles in deltamethrin resistance have not yet been systematically assess.

Interestingly, *L. salmonis* possesses only a small number of catalytically competent CaEs potentially involved in pesticide resistance (N=10), and genes encoding detoxifying ABC transporters (N=33) and CYPs (N=25) are markedly reduced too (Oakeshott et al., 2005; Carmona-Antoñanzas et al., 2015; Humble et al., 2019). In contrast, these (super)families show expansion in polyphagous or free-living ectoparasitic arthropods such as *D. melanogaster* (26 catalytically competent CaEs including 13 within the detoxification class, 56 ABC transporters, 85 CYPs) and *T. castaneum* (38 catalytically competent CaEs including 26 within the detoxification class, 73 ABC transporters, 131 CYPs), which presumably need to detoxify a wide variety of xenobiotics during their lifecycle (Broehan et al., 2013; Dean et al., 2001; Oakeshott et al., 2010). Salmon lice only ingest host products when feeding and are partially protected from environmental toxicants during host-attachment. Thus, in *L. salmonis* the reduced number of genes with roles in biochemical defence against xenobiotics may have arisen from a reduced exposure to environmental toxins (Claudianos et al., 2006; Teese et al., 2010). Similarly, the human body louse *P. humanus,* which is an obligate blood feeder, possesses only three detoxifying CaEs (in total five catalytically competent CaEs), 40 ABC transporters and 37 CYPs, and *A. mellifera*, which maintains a mutualistic symbiotic relationship with flowering plants, has only eight detoxifying CaEs (in total 13 catalytically competent CaEs), 34 ABC transporters and 46 CYPs (Claudianos et al., 2006; Lee et al., 2010). Taken together, these observations suggest that enzymes or transporters with roles in the biochemical defence only play a subordinate role in deltamethrin resistance in salmon lice.

5.4 Mechanism of resistance towards other pyrethroids

Experiments within this PhD project, as well es previous studies on *L. salmonis* focused on investigating the mechanism of resistance to deltamethrin. This raises the question whether findings are transferrable to other pyrethroids, i.e., cypermethrin.

Although deltamethrin and cypermethrin are both synthetic pyrethroids, the therapeutic margin of cypermethrin (100x) is considerably higher than that of deltamethrin (3.5x) (Roth, 2000). In addition, commercial formulations of cypermethrin and deltamethrin exist in different isomers and they have different acid moieties (Langford et al., 2015). Due to their different structure, they may act through different modes of action and their usage may has led to the selection of different resistance mechanisms. Cypermethrin was introduced in the mid-1990s, prior to the approval of deltamethrin in Norway (1998), Ireland (2007), and Scotland (2008) (Aaen et al., 2015). Resistance towards both cypermethrin and deltamethrin has already been reported in the

early 2000s (Sevatdal et al., 2005a). However, at that time resistance seemed to be unrelated to mitochondrial haplotypes (Fjørtoft et al., 2020). Only after the introduction of deltamethrin, in 2009, a mitochondrial haplotype associated with pyrethroid resistance was found in lice throughout the North Atlantic (Fjørtoft et al., 2020), which coincided with the emergence of high-level resistance towards deltamethrin in Norway (Jensen et al., 2020).

It is conceivable that the use of cypermethrin had led to the selection and enrichment of Na_v *kdr* mutation(s) and/or mechanisms involved in metabolic detoxification (Tschesche et al., 2020), which may also confer moderate levels of resistance to deltamethrin (Sevatdal et al., 2005a). However, the widespread use of deltamethrin may had favoured the selection of a stronger resistance mechanism linked to mitochondrial mutation(s) (Carmona-Antoñanzas et al., 2017; Fjørtoft et al., 2020).

Although Fjørtoft et al. (2020) first detected the deltamethrin resistance-associated mtDNA haplotype in 2009, it may have been present at very low frequencies in earlier years. It is also conceivable that the use of pyrethroids increased the mutation rate of the mtDNA according to the concept of “adaptive mutations” (Rosenberg, 2001), which may have accelerated the random appearance of resistance-conferring mtDNA SNPs. For example, the rate of mutagenic oxidation may have been increased by ROS that arose during deltamethrin induced inhibition of respiratory complexes (Alonso et al., 2003; Sipos et al., 2003), disruption of the mitochondrial membrane integrity, or intracellular Ca^{2+} accumulation (Clark and Symington, 2011; Hossain and Richardson, 2011).

5.5 Evolution and spread of deltamethrin resistance

This PhD thesis also provides insights into the evolutionary origin of deltamethrin resistance in *L. salmonis*.

As explained above, the analysis of combinations of mutations in individual parasites revealed 11 mtDNA haplotypes of which three haplotypes were associated with deltamethrin resistance. Phylogenetic analyses showed that the three deltamethrin resistance-associated haplotypes fall into two clusters, with haplotype 3 being phylogenetically distant to haplotypes 2 and 4. This mtDNA sequence variability in deltamethrin resistant isolates indicates that mtDNA-associated deltamethrin resistance in *L. salmonis* originated at form at least two independent origins, which may have been selected for more or less in parallel following the extensive use of pyrethroids. These findings contrast results by Carmona-Antoñanzas et al. (2017), who report that all analysed deltamethrin resistant isolates showed haplotype 2 and concluded that a single mitochondrial haplotype containing the resistance conferring mutation(s) must have spread geographically. However, the failure of that study to detect the less frequent haplotypes

3 and 4 can be attributed to the limited number of sequenced individuals. Similar to deltamethrin resistance, azamethiphos resistance in salmon lice has been suggested to originate from multiple independent selection events (Kaur et al., 2017). In contrast, the analysis of conserved haplotypes across samples from the Atlantic strongly suggests that emamectin benzoate resistance developed from a single source (Besnier et al., 2014).

Phylogenetic analyses described in chapter II of this PhD thesis were based on 206 genotyped samples collected in Scotland in the period 2010-2019. Thus, the data does not allow conclusions on whether the deltamethrin resistance-associated haplotypes evolved simultaneously and in multiple geographic locations. The present study also does not show whether deltamethrin resistance arose from a pre-existing neutral allele mutation under selection or from a *de novo* mutation under selection. However, Fjørtoft et al. (2020) suggested that pyrethroid resistance linked to mtDNA mutations evolved after the introduction of pyrethroids in commercial aquaculture because a resistance-associated mitochondrial haplotype first emerged around 2009, a decade after the introduction of cypermethrin and deltamethrin (Denholm et al., 2002; Grave et al., 2004). Similarly, emamectin benzoate resistance was predicted to be linked to rapid dispersal of a *de novo* mutation (Besnier et al., 2014), while the mutation conferring azamethiphos resistance in *L. salmonis* was assumed to be present at low frequencies prior to the first use of OPs in commercial aquaculture (Kaur et al., 2017).

Speculatively, the rate at which deltamethrin resistance developed may have been further accelerated by an increased mutation rate of the mtDNA. In bilaterian invertebrates, mitochondrial mutation rate estimates are ~9 times higher than those for the nuclear genomes (Brown et al., 1979; Tjensvoll et al., 2006). Increased mutation rates of mtDNA can be attributed to the following factors (Lynch 2006). First, in contrast to nuclear DNA, mtDNA is continuously replicated within nondividing cells, and the base-misincorporation rate before proofreading is 10^3 to 10^4 times greater than in the nuclear genome (Johnson and Johnson, 2001). Second, mtDNA contains fewer repair mechanisms than nuclear DNA (Croteau et al., 1999). Third, due to the close proximity to the ETC, mitochondria generate free oxygen radicals, which oxidise nucleotides and thus, may lead to mispairing events during replication (Balaban et al., 2005). Given the case that deltamethrin targets mitochondrial respiratory complex IV, mutation rates may be further increased in response to inhibitor action according to the concept of “adaptive mutations” (Rosenberg, 2001). In this context, deltamethrin induced inhibition of complex IV may increase the concentration of ROS, which may in turn increase the rate of mutagenic oxidation (Alonso et al., 2003; Sipos et al., 2003). In addition, deltamethrin treatment may increase the rate of mutagenic oxidation by ROS generated through disruption of the

mitochondrial membrane integrity or intracellular Ca^{2+} accumulation (Clark and Symington, 2011; Hossain and Richardson, 2011).

Despite the decreased usage of pyrethroids during the last decade deltamethrin resistance in *L. salmonis* is still widespread in the North Atlantic, diminishing treatment efficacies (Fjørtoft et al., 2020; Helgesen et al., 2020). This is favoured by the mode of inheritance of deltamethrin resistance in salmon lice. As explained above, evidence suggests that deltamethrin resistance in this parasite is conferred by mitochondrial genes, which are transmitted to subsequent generations through the maternal lineage. Kreitzman et al. (2018) modelled effects of dominance of nuclear genes on the development of pesticide resistance and found that the speed of evolution for resistance is fastest when resistance genes are fully dominant. Accordingly, effects of mitochondrial genes must be even stronger. Based on these considerations, resistant mitochondrial genotypes are expected to disappear only slowly, even in the absence of pesticide exposure. This is in line with findings by Fjørtoft et al. (2020), who only found a small reduction in frequency of the deltamethrin resistant mitochondrial genotype in 2017.

5.6 Detection of deltamethrin resistance in *L. salmonis*

To reduce the spread of resistance, it is important to monitor treatment efficacies and rotate treatments with different modes of action when first signs of resistance emerge (Brooks, 2009). The development of molecular resistance tests can help to monitor resistance faster, cheaper, and at much greater sensitivity than current bioassays (Aaen et al., 2015). To date, one commercial genotyping tests is available to detect resistance of *L. salmonis* towards pyrethroids. The test deems lice resistant if at least one of the following five SNPs shows the mutant genotype: T8600C, A9030G, C13953T, A14013G, and C14061T (Nilsen and Espedal, 2015). All these mutations are included in the resistance-associated haplotypes 2 and 4 identified in this PhD study, whereas resistance-associated haplotype 3 only contains SNP T8600C. When tested with all SNP markers covered by the commercial test, all resistance-associated mitochondrial haplotypes described in chapter II would be classified correctly, which explains 97% of deltamethrin resistance in the present study. However, in practice the commercial test seems to test only for SNP C14061T (Fjørtoft et al, 2019). Based on SNP C14061T, 83% of deltamethrin resistant lice would be classified correctly, while 14% of deltamethrin resistant lice with haplotype 3 would be wrongly classified as susceptible. All mitochondrial haplotypes not associated to resistance would be classified correctly.

Chapter II of this PhD thesis describes mtDNA SNP T8600C as the most accurate marker for deltamethrin resistance. Based on the data presented in this chapter, this diagnostic marker

would improve the classification of deltamethrin resistant lice by 14%. However, as explained above, a diagnostic test solely based on mtDNA markers would not be completely accurate. Regardless of the mitochondrial marker, 3% of lice rated deltamethrin resistant in bioassays were classified genotypically susceptible, suggesting additional minor roles of still unidentified nuclear genetic factors.

5.7 Fitness costs of deltamethrin resistance in *L. salmonis*

Chapter II of this PhD thesis shows that mtDNA mutations are conferring a high level of deltamethrin resistance in *L. salmonis* (EC_{50} 24.73 - 25.95 µg L^{-1}). In *H. armigera*, greater levels of resistance have been shown to come with greater fitness costs (Cao et al., 2014). Thus, a reasonable a priori expectation would be that deltamethrin resistance-conferring mtDNA mutation(s) may have implications for the fitness of the salmon louse in the absence of deltamethrin. In this context, Leu107Ser may change the COX1 structure and function and thus alter the efficiency of the respiratory chain.

Prior to selection, the mitochondrial genotype associated with deltamethrin resistance was rare (Fjørtoft et al., 2020). Between 2000 to 2019, the number of deltamethrin treatments administered in Norway peaked in 2015 (Jensen et al., 2020b). At the same time, the frequency of the deltamethrin resistant mitochondrial genotype increased (Fjørtoft, 2020). Deltamethrin usage dramatically declined after 2016 (Jensen et al., 2020b), which was mirrored by a slight decrease in resistance after 2017 (Jensen et al., 2020b). This could indicate counterselection of resistance alleles due to fitness costs.

The present PhD study did not systemically assess fitness costs of mtDNA mutations. However, in a pervious study (Carmona-Antoñanzas et al., 2017) deltamethrin susceptible and resistant control animals showed similar whole-body ATP levels, excluding severe implications on the efficiency of the respiratory chain. Furthermore, deltamethrin resistant lice from the control group showed even lower levels of apoptosis in subcuticular layer and skeletal muscle than deltamethrin susceptible lice (Bakke et al., 2018). Major fitness costs also seem to be absent as pyrethroid resistant lice were found to persist under natural conditions in regions without chemotherapeutant use (Fallang et al., 2004; Fjørtoft et al., 2020). In addition, the high diversity of the mtDNA of salmon lice from wild hosts suggests that deltamethrin resistant mitochondrial haplotypes are not associated with fitness costs. Genotyping of lice along the Norwegian coast in 2014 revealed substantially lower frequencies of the deltamethrin resistant mitochondrial genotype in lice sampled from wild salmon than from both wild sea trout and farmed salmon (Fjørtoft et al., 2019). It is unlikely that the lower frequency of the deltamethrin resistant genotype in lice from wild salmon results from fitness costs associated with higher mortality or

lower fecundity of lice carrying this marker because lice collected from wild sea trout did not show a reduced frequency of the resistant genotype. While wild salmon usually spend one to three years on offshore feeding grounds where salmon from different freshwater systems mix (Hansen and Jacobsen, 2003), wild sea trout typically utilise coastal areas for feeding where fish farms are located (Thorstad et al., 2016). Thus, a more plausible explanation would be that wild salmon returning to the Norwegian coast carry fewer deltamethrin resistant lice due to cross-infestation at offshore feeding grounds with lice originating from outside farming areas, while extensive gene flow between lice in aquaculture-dense regions explains higher frequencies of resistant lice on wild sea trout (Fjørtoft et al., 2019).

Mutation Leu107Ser may confer no/low fitness costs because the mutation is located outside the oxygen binding site and catalytic centre of the complex IV. It is also conceivable that potential adverse effects of Leu107Ser on the electron transport of the respiratory chain are compensated by significant upregulation of COX1. Accordingly, a previous study found that COX1 is the only mitochondrial gene that was significantly upregulated in resistant lice compared to susceptible lice (Bakke et al., 2018).

Taken together, further studies are needed to systematically assess potential fitness costs of resistance-associated mtDNA mutations in the absence of deltamethrin exposure in *L. salmonis*. Interestingly, in *T. urticae,* bifenazate resistance is highly correlated with mutations within cytB of ETC complex III but no fitness costs seem to incur in the absence of the pesticide (Bajda et al., 2018; Van Leeuwen et al., 2008).

5.8 Why is deltamethrin resistance in terrestrial arthropods not associated with mtDNA mutations?

Roles of mtDNA mutations in pyrethroid resistance have not been reported in terrestrial arthropods. Instead, pyrethroid resistance involves *kdr* target-site mutations in the sequence of Na_v1 (Dong et al., 2014) and/or increased metabolic detoxification (Ranson et al., 2002).

It is unlikely that mtDNA determinants of deltamethrin resistance have remained unnoticed in terrestrial arthropods. Pyrethroid resistance has been extensively studied in insect disease vectors and crop-infesting pests (Dong et al., 2014). As mutations in mitochondrial genes are inherited maternally, studies would have revealed a predominantly maternal inheritance of pyrethroid resistance. Instead, they suggest incompletely recessive inheritance of the resistance phenotype (Foil et al., 2005; Halliday and Georghiou, 1985; Hopkins and Pietrantonio, 2010; Stenhouse et al., 2013).

However, it seems conceivable that resistance mechanism involving the mitochondria could contribute to deltamethrin resistance in some terrestrial arthopods. Due to their lipophilic nature, pyrethroids can affect mitochondrial membrane structures and dynamics (Braguini et al., 2004; Zhang et al., 2007) and can induce *inhibition of mitochondrial respiratory complexes* (Braguini et al., 2004; Gassner et al., 1997). For example, the pyrethroids cyhalothrin and alpha-cypermethrin have been shown to decrease mitochondrial membrane fluidity and ATPase activity in the wolf spider (*Pirata subpiraticus*) and the rice stem borer (*Chilo suppressalis*) (Li et al., 2015). MtDNA mutations may contribute to the resistance phenotype by enhancing *kdr*-type resistance mechanisms. Similarly, pyrethroid resistance in *A. aegypti* was higher in strains with both *kdr* and CYP-mediated resistance mechanisms than in strains exhibiting *kdr*-type mutations alone (Smith et al., 2019).

In contrast to terrestrial arthropods, deltamethrin resistance in *L. salmonis* is closely linked to mutations in mitochondrial genes (Carmona-Antoñanzas et al., 2017). Different deltamethrin resistance mechanisms may be related to different target-sites. Most likely, mtDNA mutations only play a pivitol role in conferring resistance when deltamethrin toxicity is not primarily based on the blockage of Na_v1. In terrestrial arthropods, type-II pyrethroids have been shown to induce rapid knock-down effects (within 60 min in *M. domestica*) (Scott and Georghiou, 1984). In contrast, drug susceptible salmon lice exposed to deltamethrin (0.2 µg L^{-1}; 30 min) showed no signs of toxicity at the end of the exposure, whereas reassessment of the animals after further 6 h of recovery in seawater revealed 50% of lice being immobilised (Carmona-Antoñanzas et al., 2017). Similarly, Jensen et al. (2017) found that the median survival time of susceptible lice treated with the recommended treatment dosage of deltamethrin (2 µg L^{-1}; 30 min) was 16.8 h, while the corresponding median survival time for the neurotoxic compound azamethiphos was only 16 min. These results suggest deltamethrin toxicity in *L. salmonis* does not primarily involve Na_v1. Mitochondria may be an alternative target-site, which would explain the associated of mtDNA mutations with deltamethrin resistance in salmon lice.

The selective toxicity of deltamethrin between salmon lice and terrestrial arthropods may be related to differences in sodium channels. While insects produce multiple Na_v1 isoforms through alternative splicing (reviewed by Dong et al., 2017), *L. salmonis* exhibits three Na_v1 paralogues (Carmona-Antoñanzas et al., 2018). These three paralogues have been shown to be differentially expressed in different life stages/sexes and following drug exposure (Carmona-Antoñanzas et al., 2018). Cellular localisation and functional roles of the $LsNa_v1$ homologues are still unknown, but their presence may indicate subfunctionalisation such as expression in different types of neurones with heterogeneous functions in different life stages. In this case, toxic effects of deltamethrin on channel homologues may only be visible in longer time frames and/or certain

life stages. For example, deltamethrin may affect nerves involved in feeding mechanisms or compromises lice attachment by impairing mechanisms for clinging onto fish. Mitochondria may have gained in importance as an alternative target-site. However, more research is needed to resolve functions of Na_v1 paralogues in *L. salmonis*.

MtDNA mutations may also be of no or lower importance in conferring deltamethrin resistance in terrestrial arthropods due to fitness costs. In *L. salmonis*, fitness costs assoiated with mutations in mitochondrial genes cannot be ruled out (see section 5.7). However, as discussed in section 5.3.3, salmon lice have a reduced number of genes with roles in metabolic detoxification of xenobiotics and *kdr*-type mutations may be of minor importance due to subfunctionalisation of $LsNa_v1$ homologues. Thus, the absence of alternative resistance strategies may have let to the selection of mitochondrial resistance mechanisms in salmon lice, despite potential fitness costs.

It is also conceivable that different deltamethrin resistance mechanisms have been identified due to different treatment strategies. Agricultural pests are typically controlled by high-dose strategies, as arthropod-specific targets are lacking in plants. In contrast, parasitic pests have to be treated with stricter dose limitations to avoid detrimental effects in the host (Aaen et al., 2015). Maybe high-dose strategies, as used to control agricultural pests, are necessary to select for *kdr* resistance mechanisms or to induce protective effects of *kdr* mutations. In contrast, mitochondrial resistance mechanism may only be observable in the lower-dose strategies used to control salmon lice. However, previous findings are inconclusive as salmon lice with deltamethrin resistance-associated mtDNA mutations can be highly resistant, i.e., can survive deltamethrin concentrations that would kill their host fish (Carmona-Antoñanzas et al., 2017). Thus, further studies are needed to validate this hypothesis.

Comparison between deltamethrin resistance mechanisms in terrestrial arthropods and salmon lice is further complicated by resistance being a moving target (Ffrench-Constant, 2013). For example, in *D. melanogaster* DDT resistance has been linked to an adaptive series of Cyp6g1 alleles that varied across time and space (Schmidt et al., 2010).

Taken together, further studies are needed to systematically assess potential roles of mtDNA mutations in deltamethrin resistance in terrestrial arthropods.

5.9 Conclusion

The overarching aim of this PhD thesis was to identify molecular determinants for deltamethrin resistance in *L. salmonis* and to obtain insights into the underlying mechanisms of resistance.

Chapter I investigated the relative importance of Na_v1 target-site mutations as compared to mtDNA mutations as determinants of deltamethrin resistance in *L. salmonis*. Results obtained from this study provided no evidence for roles of classical target-site mutations in Na_v1. A deltamethrin resistant *L. salmonis* strain did not show cross-resistance to etofenprox, a compound that has been used to detect target-site resistance based on Na_v1 mutations. Moreover, mutation I936V $LsNa_v1.3$, which is homologous to a previously characterised *kdr* mutation in insects, showed no association with deltamethrin resistance in *L. salmonis* from farm sites, as defined based on the results of acute toxicity tests carried out with adult/preadult parasites. Thus, hypothesis 1 must be rejected.

To disentangle roles of nuclear and mitochondrial mutations in pyrethroid resistance, the study presented in chapter I further tested previously identified mtDNA SNPs as genetic markers for resistance in the field (Carmona-Antoñanzas et al., 2017; Nilsen and Espedal, 2015). In contrast to $LsNa_v1.3$ I936V, the mitochondrial SNPs significantly differentiated deltamethrin resistant and susceptible parasites from farm sites. This suggests that deltamethrin resistance in *L. salmonis* involves mitochondrial genetic determinants and can be reliably monitored by mitochondrial SNP markers (Carmona-Antoñanzas et al., 2017).

Chapter II of this PhD thesis investigated the relationship between mitochondrial SNPs and deltamethrin resistance further, aiming to obtain deeper insights into the involvement of mitochondrial SNPs in the resistance mechanism and to pinpoint the most accurate deltamethrin resistance marker. *L. salmonis* from farm sites, laboratory strains, and wild hosts were characterised regarding their deltamethrin susceptibility and genotyped at selected mitochondrial SNP loci to identify mitochondrial haplotypes and assess haplotype association with deltamethrin resistance (Carmona-Antoñanzas et al., 2017). Results revealed that deltamethrin resistance in *L. salmonis* is associated with multiple mtDNA haplotypes which have multiple origins. Comparison of mtDNA sequence between well-characterised resistant and susceptible *L. salmonis* strains showed that mtDNA SNP T8600C, corresponding to Leu107Ser in COX1, was the only non-synonymous mutation common to all resistant lice but lacking in all susceptible lice. Parasites of an isolated laboratory strain in which T8600C was the only non-synonymous mutation were highly deltamethrin resistant and passed on their resistance to the next generation through maternal inheritance. These results suggest the association of

deltamethrin resistance with SNP T8600C (Leu107Ser in COX1). Thus, hypothesis 2 can be accepted.

In terrestrial arthropods, deltamethrin resistance can also be conferred by detoxifying enzymes such as CaEs (reviewed by Oakeshott et al., 2005). Chapter III of this PhD thesis characterised the CaE family in salmon lice and investigated its potential role in resistance to deltamethrin. The study revealed that the CaE gene family of *L. salmonis* is one of the smallest characterised in arthropods to date. It includes catalytically inactive proteins predicted to be involved in neurodevelopmental function as well as secreted catalytically competent enzymes. In addition, the *L. salmonis* CaE gene family contains a new clade, which is predicted to be largely catalytically competent and soluble. However, analysis of transcript expression and SNPs suggested that CaEs are not major determinants of deltamethrin resistance. Thus, hypothesis 3 must be rejected. Nonetheless, CaEs could still have relevance as factors contributing to the resistance phenotype.

Taken together, results from the present PhD study suggest that deltamethrin resistance, and by inference potentially the mechanism of deltamethrin toxicity, differ between *L. salmonis* and terrestrial arthropods. In *L. salmonis*, deltamethrin resistance is inherited maternally, and resistance seems to be associated with mutations in the mtDNA, most likely SNP T8600C corresponding to Leu107Ser in COX1. Results also suggest additional minor roles of nuclear genetic factors. However, no clear evidence was found for roles of classical target-site mutations in Na_v1 and CaE genes in mediating deltamethrin resistance.

5.10 Future perspectives

Sea lice infections are a major health management problem in the commercial mariculture of Atlantic salmon. In 2018, the estimated global costs of sea lice infections to the salmon industry were approximately USD $873 million / GBP £700 million, comprising mainly treatment costs and to a lesser extent losses in production (Brooker et al., 2018b).

To date, the control of sea lice still relies on veterinary drugs (Jensen et al., 2020), and as long as that is the case resistance development towards these drugs will be a constant concern. Molecular tests can help to monitor drug resistance faster, cheaper, and at much greater sensitivity than current bioassays (Aaen et al., 2015). They can be used both to describe the current situation of resistance and to evaluate the effect of measures taken to slow the speed of resistance development. Moreover, close monitoring of treatment efficacies enables to rotate drugs with different modes of action as soon as first signs of resistance emerge and, thereby, reduces the amount of chemicals used (Brooks, 2009). The present PhD project identified SNP T8600C as the most reliable diagnostic marker for deltamethrin resistance in salmon lice. This

marker is 14% more accurate in identifying deltamethrin resistant lice than a currently available commercial test based on other mtDNA markers T8600C, A9030G, C13953T, A14013G, or C14061T. For example, T8600C could replace less accurate markers for deltamethrin resistance in the resistance surveillance program of the Norwegian Food Safety Authority, which monitors the distribution of resistance along the Norwegian coast (Helgesen et al., 2020). However, a diagnostic test solely based on mtDNA markers is not completely accurate. Regardless of the mitochondrial marker, 3% of lice rated deltamethrin resistant in bioassays were classified genotypically susceptible, suggesting additional minor roles of nuclear genetic factors in resistance.

According to this PhD project, Na_v1 target-site mutations seem to have no or only secondary relevance in conferring deltamethrin resistance in adult and pre-adult *L. salmonis* and no clear evidence was found for a role of CaE genes in mediating resistance (Tschesche et al., 2020, 2021). Thus, the exact molecular determinants for deltamethrin resistance in salmon lice could not yet be pinpointed. For example, potential roles of serine proteases, GSTs, and ABC transporters in pyrethroid resistance remain to be investigated. Moreover, it remains to be proven whether T8600C is causally linked to deltamethrin resistance in salmon lice.

Further experiments could be designed to delineate the resistance mechanisms studied in this PhD: Electrophysiological characterization of the $LsNa_v1$ homologues in *Xenopus* oocytes could be performed to study the role of putative *kdr* mutations in reducing the channel's sensitivity to deltamethrin (Usherwood et al., 2007). The deltamethrin metabolism by hydrolysing enzymes could be investigated in resistant and susceptible salmon lice by measuring the concentration of the compound and its main metabolites DA and 3-PBA using liquid chromatography coupled with tandem mass spectrometry (Ikonomou and Surridge, 2013). The effect of deltamethrin on mitochondrial function could, for example, be assessed by measuring the oxygen consumption rate of isolated mitochondria from resistant and susceptible lice using a Clark-type electrode or high-resolution respirometry (Lanza and Nair, 2009). A future approach may involve the use of mitochondrial gene editing tools, such as the CRISPR-Cas9 system, to edit the COX1 gene that contains T8600C (Yang et al. 2021).

When the molecular mechanisms for deltamethrin resistance are established, they can be further developed into high-throughput resistance assays, which would increase the accuracy of a diagnostic test solely based on SNP T8600C.

Knowledge of resistance mechanisms may also prove useful to develop new medicines that are not combated by already existing resistance mechanisms. To avoid cross-resistance, it is important to apply chemicals that select for different resistance-conferring mutations (Coates et

al., 2021b). If the resistance mechanism is a type of metabolic resistance, synergists may be added to the treatment protocol to inhibit the mechanism (Silcox et al., 1985).

In this PhD project, analyses were restricted to *L. salmonis* samples from Scotland. Although there is little evidence for population genetic differentiation among *L. salmonis* throughout the entire North Atlantic Ocean (Besnier et al., 2014; Glover et al., 2011), results should be validated with field samples from other North Atlantic regions, e.g., Norway. Moreover, it would be beneficial to investigate the relationship between deltamethrin resistance and mtDNA haplotypes not only in Scotland but across the North Atlantic. This would give a better picture of the evolution of deltamethrin resistance. Findings would be even more conclusive if archived samples from before and after the introduction of deltamethrin in commercial aquaculture would be genotyped and compared.

So far, studies investigating the molecular mechanism of deltamethrin resistance in caligid sea lice have been limited to *L. salmonis*. Studies on *C. rogercresseyi*, the most prevalent sea lice species in the Southern hemisphere (Johnson and Jakob, 2012), are comparatively scarce (Núñez-Acuña et al., 2020). If deltamethrin resistance in *C. rogercresseyi* is based on similar mechanisms than in *L. salmonis*, diagnostic test for resistance could be developed comparatively quick by methods employed within this PhD project.

Whether deltamethrin resistance is associated with fitness cost in salmon lice is not yet fully understood. Fitness costs would counteract the spread of resistance in the absence of deltamethrin (Coustau and Chevillon, 2000), a process that would increase the importance of refugia for sensitive parasites and area fallowing as part of a resistance management strategy.

Resistance testing is only a small part of the bigger picture in gaining control over salmon lice. For most effective control, a multifaceted and systematic resistance management strategy is required. In this regard, an integrated pest management strategy has been developed, which combines a variety of tools for most effective sea lice control. Key elements are lice level monitoring, husbandry and management, prevention, and resistance monitoring and intervention (Brooks, 2009). Emphasis should be placed on preventive measures rather than reactionary techniques (Barrett et al., 2020). To date, comprehensive resistance monitoring is limited to the Norwegian coastline (Helgesen et al., 2020). To obtain a better picture of the severity and development of resistance, it would be beneficial to collecting the same data in the rest of the salmon producing world. For sustainable sea louse control, coordination between farms across the louse population is important. For example, farms in the same area should avoid using concurrent strategies because this can accelerate louse adaptation and cross-resistance.

As explained above, the development of both new anti-parasitic drugs with different modes of action and non-medicinal control methods would help to slow the spread of resistance by increasing the number of tools available to combat sea lice infestations. However, it must be noted that evolution of resistance is still conceivable if treatments are not 100% efficient, genetic determinants exist that increase parasite fitness in the presence of treatments, and treatments are implemented in a way providing sufficient selection pressure. For example, there are concerns that lice may adapt resistance to fresh and warm water treatments (Andrews and Horsberg, 2021, 2020; Ljungfeldt et al., 2017), barrier technologies (Coates et al., 2021a, 2020), and cleaner fish (Hamre et al., 2021). However, while chemical resistance can arise through mutations of a small number of genes, non-chemical resistance may require more complex changes to anatomy, physiology, and behaviour and thus, may come with greater trade-offs and may take longer to arise (Coates et al., 2021b). Taken together, it is of major importance to implement a wide variety of control methods in an optimal manner.

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Supplementary Material

Table S1.1 Criteria for behavioural rating of preadult-II/adult *L. salmonis* in bioassays.

Reference	Criteria
Carmona-Antoñanzas et al., 2016	**Live:** Firmly attached to the surfaces of the Petri dish or swimming normally. **Weak:** Swimming irregularly and failing to attach to surfaces firmly (animals may attach briefly but dislodge again instantly). **Moribund:** Incapable of swimming away or attaching (animals may twitch appendages or move uncoordinatedly in a close circle). **Dead:** No movements in extremities, gut, or other organs as apparent from examination under a microscope.
Sevatdal and Horsberg, 2003	**Live:** Attached to the walls of the box or displaying an active swimming behaviour. **Moribund:** Not capable of attaching to a surface using the cephalothorax as a ‘sucking disc. Movements of limbs or internal organs could still be observed. **Dead:** No movements in extremities, gut or other organs could be noted.
Westcott et al., 2008	**Live:** Normal swimming behaviour; Securely adheres to Petri dish; Normal movement of extremities. **Moribund:** Disabled swimming but capable of weak uncoordinated movement; Inability to firmly adhere to Petri dish; Minimal movement of extremities. **Dead:** Inability to swim; Floating in Petri dish; No movement of extremities.
Igboeli et al., 2012	**Live:** See criteria according to Westcott et al. (2008). **Weak:** Poor and irregular swimming; unable to attach to the Petri dish. **Moribund:** Immotile with twitching appendages. **Dead:** See criteria according to Westcott et al. (2008).
Saksida et al., 2013	**Live:** Good swimmers attach to sides of Petri dish and remain in place, resist pulling off. **Weak:** Swim and flip, but within seconds of attaching to the side of the Petri dish they fall. **Moribund:** No swimming, animals twitch abdominal or thoracic appendages in response to gentle prodding with forceps. **Dead:** No response to prodding, no signs of life.
Helgesen and Horsberg, 2013	1. After 24 h recovery flasks were turned upside down three times and rotated in a circle 10 times. 2. Content was rapidly poured out through a funnel containing a filter. 3. 25% of the solution was returned to flask and procedure was repeated. **Alive:** Lice that were still attached to the walls of the glass bottles or the beaker and those that swam in a straight line. **Inactive/dead:** The rest of the lice.

Table S1.2 Conditions in bioassays with preadult-II and adult *L. salmonis*.

Reference	Compounds and solvent	Bioassay set-up	Exposure temperature	Exposure time | SW recovery (Number of concentrations including SW control)
Sevatdal and Horsberg, 2003	DTM: AlphaMax in SW	- **Exposure in perforated polystyrene boxes placed in aerated SW** - At least duplicates	12°C	- DTM: 30 min | 6, 24, 48 h - Eight concentrations
Helgesen and Horsberg, 2013	- DTM: AlphaMax in SW - AZA: AZA in ethanol - EMB: EMB in ethanol		12°C	- DTM: 30 min | 24 h - AZA: 60 min | 24 h - EMB: 24 h | - - Six concentrations
Helgesen et al., 2015	- Hydrogen peroxide: Paramove in SW		12°C	- Hydrogen peroxide: 30 min | 24 h - Six to twelve concentrations
Sevatdal et al., 2005	- Cypermethrin: Excis in SW - High-cis-cypermethrin: Betamax in SW - DTM: AlphaMax in SW		n.d.	- Cypermethrin: 60 min |24 h - High-cis-Cypermethrin: 30 min |24 h - DTM: 30 min |24 h - Six concentrations
Sevatdal and Horsberg, 2003	DTM: AlphaMax in SW	- **Exposure on salmon** - One salmon with 9-33 lice exposed in tank containing 30 L SW - Duplicates	12°C	- DTM: 30 min | 6, 24, 48 h - Fish rinsed in FW and lice and fish placed in 20 L tanks - Fish killed 1 h after exposure - Attached and detached lice transferred into separate perforated polystyrene boxes - Six concentrations
Helgesen and Horsberg, 2013; Helgesen et al., 2015	- DTM: AlphaMax in SW - AZA: AZA in ethanol - EMB: EMB in ethanol	- **Exposure in 1 L glass flasks** - Containing 1000 mL SW	12°C	- DTM: 24 h | - - AZA: 24 h | - - EMB: 24 h | - - Hydrogen peroxide: 24 h | - - Different concentrations tested to identify the biggest difference between the lower 80% PI for the susceptible strain and the upper 80% PI for the resistant strain
Westcott et al., 2008; Carmona-Antoñanzas et al., 2016	EMB: EMB in methanol	- **Exposure in Petri dishes** - 10-15 lice per dish - At least duplicates	10°C	- EMB: 24 h | - - At least six concentrations)
Igboeli et al., 2012; Saksida et al., 2013	EMB: EMB in methanol or PEG300		10°C	- EMB: 24 h | - - Six concentrations
Carmona-Antoñanzas et al., 2016	EMB: EMB in PEG300		12°C	- Hourly examinations 1-15 h after addition of toxicants, followed by examinations at 18, 21, and 24 h - Four concentrations

SW: seawater; DTM: deltamethrin; EMB: emamectin benzoate; AZA: azamethiphos; n.d.: n.d. in publication, PI: prediction interval.

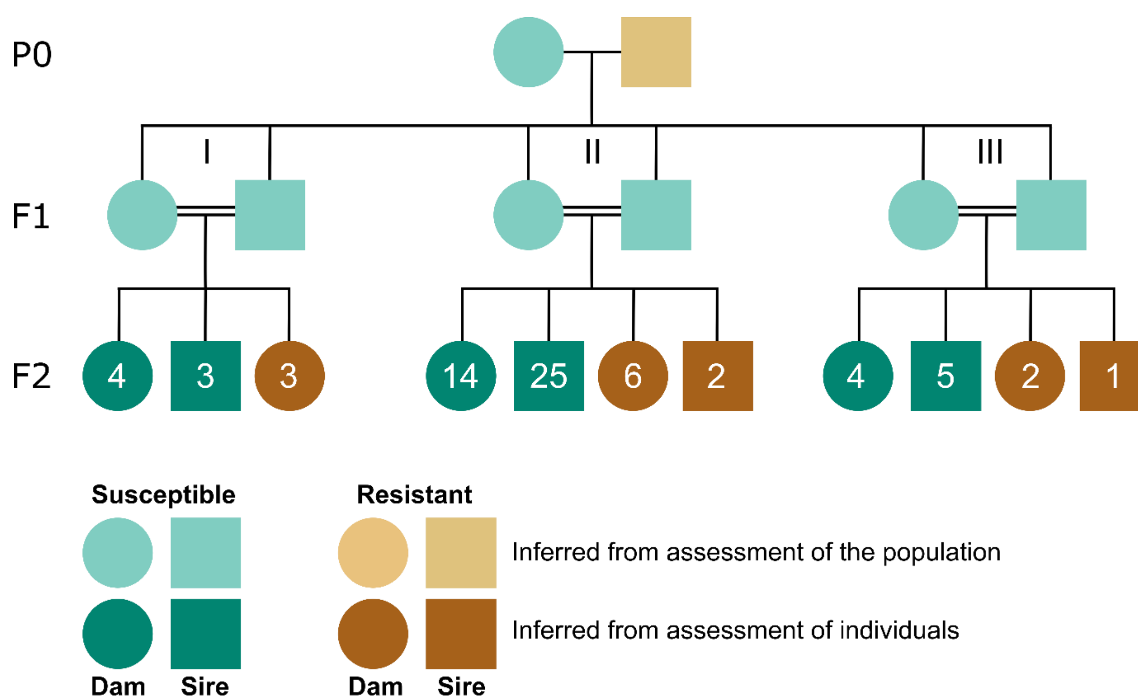

Figure S2.1 *Lepeophtheirus salmonis* cross spanning three generations. The family was initiated at the parental generation P0 by setting up a breeding pair consisting of a deltamethrin resistant male from strain IoA-02 and a susceptible female from strain IoA-00. Sibling crosses were set up between F1 progenies to produce F2 progenies. F1 pairs and their offspring are considered subfamilies and labelled by Roman numerals. The deltamethrin susceptibility of F2 progenies was determined in single-dose bioassays, involving exposure (30 min) to 2 µg L^{-1} deltamethrin, followed by recovery in seawater (24 h) and subsequent rating as susceptible (affected) or resistant (unaffected). Deltamethrin susceptibility of P0 parasites was assumed to correspond to the drug susceptibility of their strains of origin, with the IoA-02 male being considered resistant and the IoA-00 female being considered susceptible. F1 individuals not required to propagate crosses were pooled and subjected to standard deltamethrin bioassays. Numbers within symbols are the number of resistant or susceptible F2 males or females within each subfamily.

Table S2.1 Raw data used to determine median effective concentrations (EC_{50}).

Date of experiment	Origin of *L. salmonis*	Active ingredient	Solvent/ Formu-lation	Sex	Drug concen-tration (µg L^{-1})	Total number parasites	Number affected parasites
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	0	11	0
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	0	11	0
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	0.1	11	0
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	0.1	11	0
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	0.3	11	2
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	0.3	11	3
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	1	11	3
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	1	11	4
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	3	11	7
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	3	11	7
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	10	11	9
03/05/2017	Sutherland	DTM	AlphaMax	n.d.	10	11	11

Date of experiment	Origin of *L. salmonis*	Active ingredient	Solvent/ Formu-lation	Sex	Drug concen-tration (µg L^{-1})	Total number parasites	Number affected parasites
26/11/2018	Argyll	DTM	Acetone	n.d.	0	32	0
26/11/2018	Argyll	DTM	Acetone	n.d.	2	54	3
19/05/2019	Argyll	DTM	AlphaMax	n.d.	0	15	1
19/05/2019	Argyll	DTM	AlphaMax	n.d.	0	15	0
19/05/2019	Argyll	DTM	AlphaMax	n.d.	0	14	2
19/05/2019	Argyll	DTM	AlphaMax	n.d.	2	15	2
19/05/2019	Argyll	DTM	AlphaMax	n.d.	2	15	3
19/05/2019	Argyll	DTM	AlphaMax	n.d.	2	14	2
19/05/2019	Argyll	DTM	AlphaMax	n.d.	4	15	3
19/05/2019	Argyll	DTM	AlphaMax	n.d.	4	15	2
19/05/2019	Argyll	DTM	AlphaMax	n.d.	4	14	2
19/05/2019	Argyll	DTM	AlphaMax	n.d.	6	15	5
19/05/2019	Argyll	DTM	AlphaMax	n.d.	6	15	6
19/05/2019	Argyll	DTM	AlphaMax	n.d.	6	14	6
19/05/2019	Argyll	DTM	AlphaMax	n.d.	10	15	11
19/05/2019	Argyll	DTM	AlphaMax	n.d.	10	15	9
19/05/2019	Argyll	DTM	AlphaMax	n.d.	10	14	8
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	0	15	0
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	0	15	1
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	2	15	6
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	2	15	4
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	4	15	11
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	4	15	11
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	6	15	13
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	6	15	13
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	10	15	14
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	10	15	14
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	16	15	14
06/09/2019	Sutherland	DTM	AlphaMax	n.d.	16	15	13
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0	10	5
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0	10	0
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0	10	0
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0.2	10	0
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0.2	10	2
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0.2	10	2
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0.6	10	1
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0.6	10	3
19/04/2019	Inverness	DTM	AlphaMax	n.d.	0.6	10	4
19/04/2019	Inverness	DTM	AlphaMax	n.d.	2	10	0
19/04/2019	Inverness	DTM	AlphaMax	n.d.	2	10	3
19/04/2019	Inverness	DTM	AlphaMax	n.d.	2	10	4
19/04/2019	Inverness	DTM	AlphaMax	n.d.	6	10	8
19/04/2019	Inverness	DTM	AlphaMax	n.d.	6	10	2
19/04/2019	Inverness	DTM	AlphaMax	n.d.	6	10	7
19/04/2019	Inverness	DTM	AlphaMax	n.d.	20	10	10

Date of experiment	Origin of *L. salmonis*	Active ingredient	Solvent/ Formu-lation	Sex	Drug concen-tration (µg L^{-1})	Total number parasites	Number affected parasites
19/04/2019	Inverness	DTM	AlphaMax	n.d.	20	10	9
19/04/2019	Inverness	DTM	AlphaMax	n.d.	20	10	10
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0.046	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0.046	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0.1	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0.1	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0.215	5	1
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0.215	5	1
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0.464	5	2
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	0.464	5	2
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	1	5	5
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	1	5	5
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	2.154	5	5
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	2.154	5	5
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	4.624	5	5
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Female	4.624	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0.046	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0.046	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0.1	6	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0.1	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0.215	4	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0.215	5	1
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0.464	5	1
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	0.464	4	1
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	1	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	1	5	4
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	2.154	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	2.154	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	4.624	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Female	4.624	6	6
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0.046	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0.046	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0.1	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0.1	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0.215	5	0
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0.215	5	2
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0.464	5	3
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	0.464	5	5
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	1	5	5

Date of experiment	Origin of *L. salmonis*	Active ingredient	Solvent/ Formu-lation	Sex	Drug concen-tration (µg L^{-1})	Total number parasites	Number affected parasites
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	1	5	5
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	2.154	5	5
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	2.154	4	4
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	4.624	6	6
11/05/2018	Strain IoA-00	Etofenprox	Ethanol	Male	4.624	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0.046	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0.046	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0.1	4	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0.1	5	1
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0.215	5	1
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0.215	5	0
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0.464	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	0.464	6	6
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	1	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	1	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	2.154	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	2.154	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	4.624	5	5
11/05/2018	Strain IoA-02	Etofenprox	Ethanol	Male	4.624	5	5

DTM: Deltamethrin, n.d.: not defined.

Table S2.2 *Lepeophtheirus salmonis* primer sequences used for allele-specific PCR assays (KASP®).

Name	Primer allele 1 (FAM)	Primer allele 2 (HEX)	Common primer	Allele 1	Allele 2
Na_v1.3 3041	GAAGGTGACCAAGTTCATGCTCCAATTTGACTTTTGTTCTCTGTATTATCA	GAAGGTCGGAGTCAACGGATTCAATTTGACTTTTGTTCTCTGTATTATCG	CTTCCCAAAGAGTTGCATCCCCATA	A	G
CytB 14013	TCTTTTATCCCCCAGCAAAAATGGA	CTTTTATCCCCCAGCAAAAATGGG	GTAGTGGCTTTAGCTTTGTCTGTAAGAAT	A	G
COX1 9030	TTATATTCTAATTCTTCCAGGGTTTGGA	ATATTCTAATTCTTCCAGGGTTTGGG	GCCTCATCTTTACAAGTTTCTTGGGTAAT	A	G

Na_v1.3: Voltage-gated sodium channel homologue 1.3, CytB: Cytochrome b, COX1: Cytochrome c oxidase.

Table S3.1 Deltamethrin concentrations (µg L^{-1}) used in bioassays *L. salmonis* with first filial (F1) progenies derived from parental (P0) crosses of different gender-strain orientations.

	Deltamethrin concentration (µg L^{-1})									
P0 generation	**0.0**	**0.125**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**16.0**	**32.0**
IoA-10 dam x IoA-10 sire	x	x	x	x	x	x	x	x	x	x
IoA-00 dam x IoA-00 sire	x	x	x	x	x	x	x			
IoA-02 dam x IoA-02 sire	x				x	x	x	x	x	x
IoA-00 dam x IoA-10 sire	x	x	x	x	x	x	x	x	x	x
IoA-10 dam x IoA-00 sire	x	x	x	x	x	x	x	x	x	x

Table S3.2 *Lepeophtheirus salmonis* primer sequences used for allele-specific PCR assays (KASP®).

Name	Primer for allele 1 (FAM)	Primer for allele 2 (HEX)	Common primer	Allele 1	Allele 2
3338	AATAGGCCTAAAGCCACAAACC	CCTAATAGGCCTAAAGCCACAAACT	GTCTCCTTGGCCTCTATTAAGGTCTT	G	A
5889	AAAGTGGGAAGTGTATCAAGGCTTTT	AAGTGGGAAGTGTATCAAGGCTTTC	CTGGTAAAATTTAAAAGACCCTCCTCAATT	T	C
8134	TTTGTTTTATGTAATTAGAAGGTTAGGAG	ACTTTTGTTTTATGTAATTAGAAGGTTAGGAA	TAACCCGCCTTCACACTCACAAGAA	G	A
8600	ACTAATGCCCTTATAAGTAATAAACTCA	ACTAATGCCCTTATAAGTAATAAACTCG	CCCCGCTTAAACAATATAAGATTTTGGTTT	T	C
714	GAGCATGTCATGAAAAGTCTAGGAGA	AGCATGTCATGAAAAGTCTAGGAGG	CACACAACTGCTCAGAGGAAGGATT	A	G
1174	CTACTATAACTTTACTCAATTTATTCCTC	CTCTACTATAACTTTACTCAATTTATTCCTT	TTAAGGCAATCGAGATTGGAAGTAGGAAA	G	A
1678	ATAGACCCCATAAGAGGGGCC	ATAGACCCCATAAGAGGGGCT	TACATTTATGACTTCCCTTAGCTCACGTA	C	T
3056	GTGTTATACTGTTGTAGGGTTATACTC	GAGTGTTATACTGTTGTAGGGTTATACTT	GTTCGTCTTCCGATTAATTTCAGCTGTAA	C	T
4563	CACACCTTAAGTATCTTTCTACCCTC	ACACACCTTAAGTATCTTTCTACCCTT	TTTCTAAGTGTAGGAGGAGTGGGGA	G	A
6325	GAGGAACAATTACCCCCTGAGC	GAGGAACAATTACCCCCTGAGT	GGTAACTTAAGGTATTCTGAACTAATCTTA	G	A
9030	TTATATTCTAATTCTTCCAGGGTTTGGA	ATATTCTAATTCTTCCAGGGTTTGGG	GCCTCATCTTTACAAGTTTCTTGGGTAAT	A	G
9426	CCACTTTCACTATGTTCTTAGAATAGGA	CACTTTCACTATGTTCTTAGAATAGGG	AGTGAACCCCGCTATTAGGGCAAAA	A	G
10094	CCTATGTGTAGCAAAATAATTGGTTCAG	ACCTATGTGTAGCAAAATAATTGGTTCAA	GGCTGGGATAGCCCACTTAATATAATAAA	G	A
10722	CCGATTAAAAATATCTATCCTCTCTCTC	CCCGATTAAAAATATCTATCCTCTCTCTA	CGTGTTCATCCTTGTCTTATAGCTTAGTT	TAG	T
11190	CCTCATGAAATGTTAATTTTTGAGATTTAAATG	CCTCATGAAATGTTAATTTTTGAGATTTAAATA	GTGCCAGCATTCGCGGTTATACTTT	C	T
13466	TAATCCGACCTCTAACCCTAAGG	GTTAATCCGACCTCTAACCCTAAGA	GCCTGCAATTATATTAGCTATAAGCCGAA	G	A
14013	TCTTTTATCCCCCAGCAAAAATGGA	CTTTTATCCCCCAGCAAAAATGGG	GTAGTGGCTTTAGCTTTGTCTGTAAGAAT	A	G
14751	CTACGGATAAAAACGCTGTAGAAATC	GTCTACGGATAAAAACGCTGTAGAAATT	CCGGAATTTTTTTGGCTATGCATTATTCAA	C	T

Table S3.3 PCR primers to amplify *L. salmonis* mtDNA sequences in six overlapping products (including PCR conditions)

PCR product	Forward primer (5' to 3')	Reverse primer (5' to 3')	Annealing temperature °C	Extension time	PCR programme	Amplicon size (nt)
1	GGGGAAAGGGGGCTATCTACTTG	TTGCTCCCGCTAACACTGGTAAA	**69**	**3 min**	**A**	**5749**
2	TGTCTCAGCCGGGAGCATATTTA	GCGTAATAACTGCCCGCATTACA	**67**	**3 min**	**A**	**5373**
3	CAAAGGTTTGCCCACCTAGTTCC	CCAAACTCCGCGGTTACTACAGA	**50/52**	**3 min**	**B**	**1810**
4	AAGCTTAGCTAATACCAGGT	ATGGTGGCTGTTACCAAAAT	**56/58**	**4 min**	**B**	**3508**
5	TTAGCTCACAATCCGTCTGA	TTAGGCCCAAACTTTTCTACT	**60**	**52 s**	**A**	**1634**
6	AGTAGAAAAGTTTGGGCCTAAA	ATATTTCTCGCCGCTGGTATATTTT	**61**	**65 s**	**A**	**1890**

Table S3.4 PCR conditions for each product.

PCR programme	Step	Temperature	Time	Cycles
A	Initial denaturation	98°C	30 s	
A	Denaturation	98°C	10 s	33
A	Annealing	See Table S3.3	20 s	
A	Extension	72°C	See Table S3.3	
A	Final extension	72°C	2 min	
B	Initial denaturation	98°C	2 min	
B	Denaturation	98°C	20 s	10
B	Annealing	See Table S3.3	30 s	
B	Extension	72°C	See Table S3.3	
B	Denaturation	98°C	20 s	20
B	Annealing	See Table S3.3	30 s	
B	Extension	72°C	See Table S3.3	
B	Final extension	72°C	2 min	

Table S3.5 Oligonucleotide primers to sequence above PCR products.

PCR product	Primer ID	Sequencing primer (5' to 3')
1	XL-PCRF1	GGGGAAAGGGGGCTATCTACTTG
	XL-PCR-R1ii	ATCTACTGAAGCCCCGGAGT
	mtDNA_Seq63F	GTTAGATGTTTCGGTTGAG
	mtDNA_Seq66F	TCTCAGTTTAGTTTCCAAGA
	mtDNA_Seq43R	CCGCTAGACCTGTTAGGAAT
	mtDNA_Seq44R	GCACAGCGTCTATTTTAACC
	mtDNA_Seq45R	TGTGAGGAATAGGGAGTTTG
	mtDNA_Seq3R	CTCCCGGAAGTAACTCAGAA
	mtDNA_Seq27R	CCTGCAGTAACCAAAGTTGA
	mtDNA_Seq26R	ATTGTACCACATCCCCTCTG
	mtDNA_Seq25R	CCCCCTTCGTCTTAAAGAAT
	CT_Seq1.1R	ACGGATCAGGGGAATATTG
	CT_Seq1.2R	TTGCCCCACTAAAAGTCC
2	XL-PCR_F2	TGTCTCAGCCGGGAGCATATTTA
	XL-PCR-F3	GAAAAGGTGCCAATGGCTGATTA
	XL-PCR_R2	GCGTAATAACTGCCCGCATTACA
	mtDNA_Seq37R	CATTGGCACCTTTTCATTGT
	mtDNA_Seq38R	GGCAAGCTTTAAATGCAAAT
	mtDNA_Seq39R	TGAGAGATAATTCTGCCATTTG
	mtDNA_Seq40R	AATCTGGTTCCCAAAGAAAT
	mtDNA_Seq41R	GGTTTCTATCTCAGGGCTTT
	mtDNA_Seq42R	ATACGCTCGAGTGTCTGAGT
	mtDNA_Seq9F	AAAAGGGGCTTTGATTTCTT
	mtDNA_Seq6F	ACTGATACCCCCTTTTGACA
3	mtDNA_Seq34R	GGGGTGGAACTTTGGGTCTT
	mtDNA_Seq35R	TCCTGCTCACATTCAACCTG
	mtDNA_Seq10F	TTAGCTCACAATCCGTCTGA
	CytBF	CAAAGGTTTGCCCACCTAGTTCC
	CytBR	CCAAACTCCGCGGTTACTACAGA
4	XL-PCR-R3.4	ATGGAAAGGGTGGCGAGTTA
	Lsal-mt14R	TGCTTACTCCTCCGTAGGTC
	mtDNA_Seq61F	AGCTAAGGGAAGTCATAAAT
	mtDNA_Seq28F	GGTCTAGGGGCTAGGGTGCT
	mtDNA_Seq17F	CTAGGAGAGAGGCTGACGAG
	mtDNA_Seq21R	GAGCTCCTCAATAGCAACAA
	CT_Seq2.1F	CAGCATTGAGTAAGCAAGC
5	mtDNA_10F	TTAGCTCACAATCCGTCTGA
	mtDNA_48R	TTAGGCCCAAACTTTTCTACT
	prom2F	CCCCAATGGATTTGAGGACCCTGTTGAATG
	prom3F	TCAGAAAGATATCTGGCCCCAGGGGAGCAC
	CT_Seq3.1F	ATCCCGCATGATATGGTC
	CT_Seq3.2F	TTCCGGTCATAATTTGGG
6	mtDNA_48F	AGTAGAAAAGTTTGGGCCTAAA
	mtDNA_56R	TTGACTTTCCAAATCTTTTA
	CT_Seq1.2F	GCTACGTTCCCCAAAGG
	CT_Seq4.1F	GCAGGGTAAAATTGACGG
	CT_Seq4.2F	AGAGTTAATTGTGGCAGGG
	CT_Seq5.2F	ATTTGGAAAGTCAAGGAGG
	CT_R1	ATATTTCTCGCCGCTGGTATATTTT
	T7	TAATACGACTCACTATAGGG
	SP6	CATTTAGGTGACACTATAG

Table S3.6 Mitochondrial haplotypes of *L. salmonis* from wild salmonids and Aquaculture production sites from different geographic areas.

Position†	Allele	1A	1B	1C	1D	1E	1F	1G	1H	2	3	4
Non-synonymous SNPs												
3338	G/A	G	G	G	G	G	G	G	G	A	G	G
5889	T/C	T	T	T	T	T	T	T	T	C	T	C
8134	G/A	G	G	G	G	G	G	G	G	A	G	A
8600	T/C	T	T	T	T	T	T	T	T	C	C	C
Synonymous SNPs												
714	A/G	A	A	A	A	A	A	A	A	G	A	G
1174	G/A	G	G	G	G	G	G	G	G	A	G	A
1678	C/T	C	T	T	C	C	C	T	C	T	C	T
3056	C/T	C	T	T	C	C	C	T	C	T	C	T
4563	G/A	G	G	G	G	G	G	A	G	A	G	A
6325	G/A	G	G	G	G	G	G	G	G	A	G	A
9030	A/G	A	A	A	A	A	A	A	G	G	A	G
9426	A/G	A	G	G	A	G	G	A	A	G	A	G
10094	G/A	G	G	G	G	G	G	G	G	A	G	A
10722	T/TAG	T	T	T	T	T	T	T	T	TAG	T	TAG
11190	C/T	C	T	T	C	C	C	T	C	T	C	T
13466	G/A	G	A	A	A	A	G	A	G	A	G	A
14013	A/G	A	A	G	A	A	A	A	A	G	A	G
14751	C/T	C	T	T	C	C	C	T	C	T	C	T

†Numbering according to the Scottish *L. salmonis* mitochondrial reference genome (NCBI accession number LT630766.1)

Table S3.7 Association of mitochondrial haplotypes with deltamethrin resistance in *L. salmonis* from wild salmonids and Aquaculture production sites from different geographic area.

Year	Origin	EC_{50} [µg L^{-1}] (95% CI)	N	Proportion of haplotypes [%] 1A	1B	1C	1D	1E	1F	1G	1H	2	3	4
2010	**Wild hosts** East Lothian	n.d.	**18**	55.6	-	5.6	5.6	5.6	5.6	5.6	5.6	-	-	11.1
2018	**Farm site 1** Argyll	>2.0†	**106**	3.8	-	-	-	-	-	-	-	73.6	15.1	7.5
2018	**Farm site 2** Argyll	>2.0‡	**54**	3.7	1.9	-	-	-	-	-	-	75.9	7.4	11.1
2019	**Farm site 3** Argyll	8.00 (5.7-10.2)§	**28**	-	-	-	-	-	-	-	-	71.4	21.4	7.1
2013	**NA01-O**	24.8 (12.2-85.7)+	**20**	-	-	-	-	-	-	-	-	70	10	20
2018	**IoA-00**	0.25 (0.2-0.3)++	**22**	68.2	31.8	-	-	-	-	-	-	-	-	-
2018	**IoA-02**	25.95 (18.0-33.9)++	**24**	-	-	-	-	-	-	-	-	100	-	-

†Single-dose bioassay: 97.2% (N_{Total}=106) remained unaffected after exposure to 2 µg L^{-1} deltamethrin.

‡Single-dose bioassay: 94.4% (N_{Total}=54) remained unaffected after exposure to 2 µg L^{-1} deltamethrin (Tschesche et al., 2019).

§EC_{50} according to Tschesche et al., 2020.

+EC_{50} according to Carmona-Antoñanzas et al., 2017.

++Raw data used to derive EC_{50} are provided in Table S3.11.

Table S3.8 Sequence variations specific to *L. salmonis* from laboratory strain IoA-10.

Position[†]	Type	Location	Description	Original	Replaced by
246	Polymorphism	Intergenic		C	T
734	Polymorphism	Intergenic		T	C
810	Deletion	Intergenic		GA	G
815	Polymorphism	Intergenic		T	C
839	Polymorphism	Intergenic		T	C
891	Insertion	Intergenic		A	AAT
895	Polymorphism	Intergenic		C	T
1175	Non-synonymous	ND4	CTC/Leu → CCC/Pro	A	G
1788	Non-synonymous	ND4	ACC/Thr → GCC/Ala	T	C
2068	Synonymous	ND4	TTG/Leu → TTA/Leu	C	T
2412	Synonymous	ND2	TTG/Leu → CTG/Leu	T	C
2643	Synonymous	ND2	CTA/Leu → TTA/Leu	C	T
3050	Synonymous	ND2	GTT/Val → GTC/Val	T	C
3540	Synonymous	COX3	TTG/Leu → TTA/Leu	G	A
4341	Synonymous	ND3	AGC/Ser → AGA/Ser	C	A
5254	Synonymous	ND5	GCT/Ala → GCC/Ala	T	C
5905	Synonymous	ND5	TCT/Ser → TCC/Ser	T	C
6122	Synonymous	ND5	CTA/Leu →TTA/Leu	C	T
6478	Polymorphism	tRNA-Met		A	G
6774	Synonymous	COX2	GGC/Gly → GGG/Gly	C	G
6870	Synonymous	COX2	GTA/Val → GTG/Val	A	G
7056	Synonymous	COX2	CGC/Arg → CGT/Arg	C	T
7398	Synonymous	ND1	CCT/Pro → CCC/Pro	T	C
7675	Synonymous	ND1	CTG/Leu → TTG/Leu	C	T
8627	Non-synonymous	COX1	GTA/Val → GCA/Ala	T	C
9619	Synonymous	COX1	TTG/Leu → CTG/Leu	T	C
10951	Polymorphism	Intergenic		A	G
12015	Synonymous	ND6	TTA/Leu → CTA/Leu	T	C
12126	Polymorphism	tRNA-Lys		A	G
12334	Synonymous	ND4L	TCC/Ser → TCT/Ser	G	A
13230	Non-synonymous	ATP6	ATT/Ile → GTT/Val	A	G
13460	Synonymous	ATP6	ACC/Thr → ACG/Thr	C	G
14502	Synonymous	CYTB	TGA/Trp → TGG/Trp	T	C
14841	Synonymous	CYTB	TCG/Ser → TCC/Ser	C	G
15384	Polymorphism	Insertion		C	T
15520	Polymorphism	Insertion		A	C

[†]Numbering according to the Scottish *L. salmonis* mitochondrial reference genome (NCBI accession no.: LT630766.1).

Table S3.9 Mitochondrial haplotypes of *L. salmonis* first filial (F1) progenies derived from parental (P0) crosses of different gender-strain orientations. Ten individuals of each cross were subjected to PCR based genotyping assays at 18 mitochondrial single nucleotide polymorphisms (SNPs), which have been described in a previous study (Carmona-Antoñanzas et al., 2017).

	Allele	IoA-02	IoA-10	IoA-00	IoA-00 dam x IoA-10 sire	IoA-10 dam x IoA-00 sire
Non-synonymous SNPs						
3338	G/A	A	G	G	G	G
5889	T/C	C	T	T	T	T
8134	G/A	A	G	G	G	G
8600	T/C	C	C	T	T	C
Synonymous SNPs						
714	A/G	G	A	A	A	A
1174	G/A	A	G	G	G	G
1678	C/T	T	C	C/T	T	C
3056	C/T	T	C	C/T	T	C
4563	G/A	A	G	G	G	G
6325	G/A	A	G	G	G	G
9030	A/G	G	A	A	A	A
9426	A/G	G	A	A/G	G	A
10094	G/A	A	G	G	G	G
10722	T/TAG	TAG	T	T	T	T
11190	C/T	T	C	C/T	T	C
13466	G/A	A	G	G/A	A	G
14013	A/G	G	A	A	A	A
14751	C/T	T	C	C/T	T	C
Haplotype		**2**	**3**	**1A/B**[†]	**1B**	**3**

[†]67 individuals were genotyped at SNPs C11190T, A9426G, and G6325A, and revealed of two haplotypes. Five individuals of each haplotype were genotypes at the remaining SNPs, which led to the identification of haplotypes 1A and 1B.

Table S3.10 Raw data of deltamethrin bioassays with copepodid *L. salmonis* larvae.

Origin	Bioassay number	Dose	Total	Unaffected	Affected
Farm site 7	13	0	3	3	0
Farm site 7	13	0.1	8	8	0
Farm site 7	13	0.1	5	5	0
Farm site 7	13	1	7	5	2
Farm site 7	13	1	7	5	2
Farm site 7	13	10	6	5	1
Farm site 7	13	10	7	2	5
Farm site 7	13	100	9	0	9
Farm site 7	13	100	5	0	5
Farm site 5	10	0	9	9	0
Farm site 5	10	0.1	9	5	4
Farm site 5	10	0.1	12	12	0
Farm site 5	10	0.22	4	3	1
Farm site 5	10	0.22	4	3	1
Farm site 5	10	0.46	6	5	1
Farm site 5	10	0.46	12	11	1
Farm site 5	10	1	9	8	1

Origin	Bioassay number	Dose	Total	Unaffected	Affected
Farm site 5	10	1	9	8	1
Farm site 5	10	2.15	11	10	1
Farm site 5	10	2.15	10	6	4
Farm site 5	10	4.64	9	5	4
Farm site 5	10	4.64	6	3	3
Farm site 5	10	10	10	5	5
Farm site 5	10	10	11	4	7
Farm site 5	10	21.54	8	3	5
Farm site 5	10	21.54	9	3	6
Farm site 5	10	46.42	7	1	6
Farm site 5	10	46.42	7	0	7
Farm site 5	10	100	9	0	9
Farm site 5	11	0	8	8	0
Farm site 5	11	0	5	5	0
Farm site 5	11	0.1	7	6	1
Farm site 5	11	0.22	9	8	1
Farm site 5	11	0.46	9	8	1
Farm site 5	11	0.46	11	9	2
Farm site 5	11	1	8	4	4
Farm site 5	11	1	11	9	2
Farm site 5	11	2.15	8	6	2
Farm site 5	11	2.15	9	6	3
Farm site 5	11	4.64	9	5	4
Farm site 5	11	4.64	9	7	2
Farm site 5	11	10	12	5	7
Farm site 5	11	10	14	6	8
Farm site 5	11	21.54	11	1	10
Farm site 5	11	21.54	10	2	8
Farm site 5	11	46.42	8	0	8
Farm site 5	11	46.42	8	0	8
Farm site 5	11	100	9	0	9
Farm site 6	12	0	7	7	0
Farm site 6	12	0.1	6	6	0
Farm site 6	12	0.22	6	6	0
Farm site 6	12	0.46	7	6	1
Farm site 6	12	1	7	7	0
Farm site 6	12	1	3	2	1
Farm site 6	12	2.15	11	7	4
Farm site 6	12	2.15	9	5	4
Farm site 6	12	4.64	11	4	7
Farm site 6	12	4.64	4	1	3
Farm site 6	12	10	12	1	11
Farm site 6	12	10	7	2	5
Farm site 6	12	21.54	8	0	8
Farm site 6	12	21.54	6	0	6
Farm site 6	12	46.42	9	0	9
Farm site 6	12	100	7	0	7

Origin	Bioassay number	Dose	Total	Unaffected	Affected
IoA-00	1	0	5	5	0
IoA-00	1	0	5	5	0
IoA-00	1	0.1	6	3	3
IoA-00	1	0.1	7	5	2
IoA-00	1	0.22	6	0	6
IoA-00	1	0.22	5	1	4
IoA-00	1	0.46	7	1	6
IoA-00	1	0.46	5	0	5
IoA-00	1	1	8	0	8
IoA-00	1	1	8	0	8
IoA-00	1	2.15	7	0	7
IoA-00	1	2.15	7	0	7
IoA-00	1	4.64	5	0	5
IoA-00	1	4.64	9	0	9
IoA-00	1	10	10	0	10
IoA-00	1	10	8	0	8
IoA-00	1	21.54	8	0	8
IoA-00	1	21.54	6	0	6
IoA-00	1	46.42	7	0	7
IoA-00	2	0	10	10	0
IoA-00	2	0	9	9	0
IoA-00	2	0.1	8	4	4
IoA-00	2	0.1	10	7	3
IoA-00	2	0.22	8	2	6
IoA-00	2	0.22	12	4	8
IoA-00	2	0.46	11	0	11
IoA-00	2	0.46	0	0	0
IoA-00	2	1	7	0	7
IoA-00	2	1	10	0	10
IoA-00	2	2.15	11	0	11
IoA-00	2	2.15	10	0	10
IoA-00	2	4.64	9	0	9
IoA-00	2	4.64	11	0	11
IoA-00	2	10	5	0	5
IoA-00	2	10	7	0	7
IoA-00	2	21.54	7	0	7
IoA-00	2	21.54	11	0	11
IoA-00	2	46.42	6	0	6
IoA-00	3	0	11	11	0
IoA-00	3	0	12	12	0
IoA-00	3	0.1	10	4	6
IoA-00	3	0.1	7	4	3
IoA-00	3	0.22	8	1	7
IoA-00	3	0.22	7	1	6
IoA-00	3	0.46	9	1	8
IoA-00	3	0.46	8	0	8
IoA-00	3	1	8	0	8

Origin	Bioassay number	Dose	Total	Unaffected	Affected
IoA-00	3	1	9	0	9
IoA-00	3	2.15	10	0	10
IoA-00	3	2.15	9	0	9
IoA-00	3	4.64	9	0	9
IoA-00	3	4.64	8	0	8
IoA-00	3	10	10	0	10
IoA-00	3	10	10	0	10
IoA-00	3	21.54	11	0	11
IoA-00	3	21.54	11	0	11
IoA-00	3	46.42	11	0	11
IoA-00	4	0	13	13	0
IoA-00	4	0	7	7	0
IoA-00	4	0.1	12	8	4
IoA-00	4	0.1	9	6	3
IoA-00	4	0.22	11	5	6
IoA-00	4	0.22	10	7	3
IoA-00	4	0.46	10	2	8
IoA-00	4	0.46	13	3	10
IoA-00	4	1	11	0	11
IoA-00	4	1	6	1	5
IoA-00	4	2.15	7	0	7
IoA-00	4	2.15	9	0	9
IoA-00	4	4.64	10	0	10
IoA-00	4	4.64	9	0	9
IoA-00	4	10	10	0	10
IoA-00	4	10	9	0	9
IoA-00	4	21.54	13	0	13
IoA-00	4	21.54	11	0	11
IoA-00	4	46.42	10	0	10
IoA-00	5	0	10	10	0
IoA-00	5	0	7	4	3
IoA-00	5	0.1	11	8	3
IoA-00	5	0.22	9	4	5
IoA-00	5	0.22	7	3	4
IoA-00	5	0.46	12	2	10
IoA-00	5	0.46	8	1	7
IoA-00	5	1	10	2	8
IoA-00	5	1	10	0	10
IoA-00	5	2.15	9	0	9
IoA-00	5	2.15	9	0	9
IoA-00	5	4.64	9	0	9
IoA-00	5	4.64	10	0	10
IoA-00	5	10	10	0	10
IoA-00	5	10	9	0	9
IoA-00	5	21.54	10	0	10
IoA-00	5	46.42	7	0	7
IoA-00	6	0	9	9	0

Origin	Bioassay number	Dose	Total	Unaffected	Affected
IoA-00	6	0.1	6	4	2
IoA-00	6	0.1	19	12	7
IoA-00	6	0.22	8	3	5
IoA-00	6	0.22	11	2	9
IoA-00	6	0.46	9	2	7
IoA-00	6	0.46	10	2	8
IoA-00	6	1	9	1	8
IoA-00	6	1	10	1	9
IoA-00	6	2.15	13	0	13
IoA-00	6	2.15	10	1	9
IoA-00	6	4.64	8	0	8
IoA-00	6	4.64	10	0	10
IoA-00	6	10	6	0	6
IoA-00	6	10	8	0	8
IoA-02	7	0	7	6	1
IoA-02	7	0	8	8	0
IoA-02	7	0.1	7	7	0
IoA-02	7	0.1	6	6	0
IoA-02	7	0.22	9	8	1
IoA-02	7	0.22	8	8	0
IoA-02	7	0.46	7	7	0
IoA-02	7	0.46	9	8	1
IoA-02	7	1	9	9	0
IoA-02	7	1	6	5	1
IoA-02	7	2.15	9	9	0
IoA-02	7	2.15	8	7	1
IoA-02	7	4.64	8	5	3
IoA-02	7	4.64	13	6	7
IoA-02	7	10	12	5	7
IoA-02	7	10	5	0	5
IoA-02	7	21.54	11	0	11
IoA-02	7	21.54	7	0	7
IoA-02	7	46.42	10	0	10
IoA-02	8	0	9	9	0
IoA-02	8	0.1	7	7	0
IoA-02	8	0.1	7	7	0
IoA-02	8	0.22	9	9	0
IoA-02	8	0.22	7	7	0
IoA-02	8	0.46	7	7	0
IoA-02	8	0.46	9	9	0
IoA-02	8	1	5	8	0
IoA-02	8	1	10	7	2
IoA-02	8	2.15	9	6	3
IoA-02	8	2.15	8	7	1
IoA-02	8	4.64	7	5	2
IoA-02	8	4.64	5	3	2
IoA-02	8	10	2	0	2

Origin	Bioassay number	Dose	Total	Unaffected	Affected
IoA-02	8	10	7	4	3
IoA-02	8	21.54	5	0	5
IoA-02	8	21.54	0	0	0
IoA-02	8	46.42	5	0	5
IoA-02	8	46.42	5	0	5
IoA-02	9	0	9	9	0
IoA-02	9	0.1	10	10	0
IoA-02	9	0.22	8	8	0
IoA-02	9	0.46	7	7	0
IoA-02	9	1	10	9	1
IoA-02	9	2.15	8	4	4
IoA-02	9	2.15	9	5	4
IoA-02	9	4.64	9	3	6
IoA-02	9	4.64	9	4	5
IoA-02	9	10	10	3	7
IoA-02	9	21.54	8	0	8

Table S3.11 Raw data of deltamethrin bioassays with preadult/adult *L. salmonis.*

Dose	Parental P0 generation	Replicate	Sex	N unaffected	N affected	N total
0	IoA-00 dam x IoA-00 sire	1	Female	6	0	6
0	IoA-00 dam x IoA-00 sire	2	Female	6	0	6
0.125	IoA-00 dam x IoA-00 sire	1	Female	6	0	6
0.125	IoA-00 dam x IoA-00 sire	2	Female	6	0	6
0.25	IoA-00 dam x IoA-00 sire	1	Female	4	1	5
0.25	IoA-00 dam x IoA-00 sire	2	Female	1	4	5
0.5	IoA-00 dam x IoA-00 sire	1	Female	0	5	5
0.5	IoA-00 dam x IoA-00 sire	2	Female	2	4	6
1	IoA-00 dam x IoA-00 sire	1	Female	0	5	5
1	IoA-00 dam x IoA-00 sire	2	Female	0	5	5
2	IoA-00 dam x IoA-00 sire	1	Female	0	6	6
2	IoA-00 dam x IoA-00 sire	2	Female	0	6	6
4	IoA-00 dam x IoA-00 sire	1	Female	0	6	6
4	IoA-00 dam x IoA-00 sire	2	Female	0	6	6
0	IoA-00 dam x IoA-00 sire	1	Male	5	0	5
0	IoA-00 dam x IoA-00 sire	2	Male	5	0	5
0.125	IoA-00 dam x IoA-00 sire	1	Male	4	1	5
0.125	IoA-00 dam x IoA-00 sire	2	Male	2	2	4
0.25	IoA-00 dam x IoA-00 sire	1	Male	5	0	5
0.25	IoA-00 dam x IoA-00 sire	2	Male	1	4	5
0.5	IoA-00 dam x IoA-00 sire	1	Male	0	6	6
0.5	IoA-00 dam x IoA-00 sire	2	Male	0	4	4
1	IoA-00 dam x IoA-00 sire	1	Male	0	5	5
1	IoA-00 dam x IoA-00 sire	2	Male	0	5	5
2	IoA-00 dam x IoA-00 sire	1	Male	0	3	3
2	IoA-00 dam x IoA-00 sire	2	Male	0	4	4
4	IoA-00 dam x IoA-00 sire	1	Male	0	4	4

Dose	Parental P0 generation	Replicate	Sex	N unaffected	N affected	N total
4	IoA-00 dam x IoA-00 sire	2	Male	0	6	6
0	IoA-00 dam x IoA-10 sire	1	Female	4	0	4
0	IoA-00 dam x IoA-10 sire	2	Female	4	0	4
0.125	IoA-00 dam x IoA-10 sire	1	Female	5	1	6
0.125	IoA-00 dam x IoA-10 sire	2	Female	4	0	4
0.25	IoA-00 dam x IoA-10 sire	1	Female	4	1	5
0.25	IoA-00 dam x IoA-10 sire	2	Female	5	0	5
0.5	IoA-00 dam x IoA-10 sire	1	Female	3	2	5
0.5	IoA-00 dam x IoA-10 sire	2	Female	1	4	5
1	IoA-00 dam x IoA-10 sire	1	Female	0	5	5
1	IoA-00 dam x IoA-10 sire	2	Female	3	1	4
2	IoA-00 dam x IoA-10 sire	1	Female	0	5	5
2	IoA-00 dam x IoA-10 sire	2	Female	1	4	5
4	IoA-00 dam x IoA-10 sire	1	Female	0	5	5
4	IoA-00 dam x IoA-10 sire	2	Female	0	5	5
8	IoA-00 dam x IoA-10 sire	1	Female	0	5	5
8	IoA-00 dam x IoA-10 sire	2	Female	0	6	6
16	IoA-00 dam x IoA-10 sire	1	Female	0	5	5
16	IoA-00 dam x IoA-10 sire	2	Female	0	5	5
32	IoA-00 dam x IoA-10 sire	1	Female	0	7	7
32	IoA-00 dam x IoA-10 sire	2	Female	0	3	3
0	IoA-00 dam x IoA-10 sire	1	Male	6	0	6
0	IoA-00 dam x IoA-10 sire	2	Male	6	0	6
0.125	IoA-00 dam x IoA-10 sire	1	Male	6	0	6
0.125	IoA-00 dam x IoA-10 sire	2	Male	6	0	6
0.25	IoA-00 dam x IoA-10 sire	1	Male	6	1	7
0.25	IoA-00 dam x IoA-10 sire	2	Male	3	1	4
0.5	IoA-00 dam x IoA-10 sire	1	Male	2	3	5
0.5	IoA-00 dam x IoA-10 sire	2	Male	2	4	6
1	IoA-00 dam x IoA-10 sire	1	Male	0	4	4
1	IoA-00 dam x IoA-10 sire	2	Male	4	2	6
2	IoA-00 dam x IoA-10 sire	1	Male	0	5	5
2	IoA-00 dam x IoA-10 sire	2	Male	1	4	5
4	IoA-00 dam x IoA-10 sire	1	Male	0	5	5
4	IoA-00 dam x IoA-10 sire	2	Male	0	6	6
8	IoA-00 dam x IoA-10 sire	1	Male	0	6	6
8	IoA-00 dam x IoA-10 sire	2	Male	0	5	5
16	IoA-00 dam x IoA-10 sire	1	Male	0	5	5
16	IoA-00 dam x IoA-10 sire	2	Male	0	6	6
32	IoA-00 dam x IoA-10 sire	1	Male	0	5	5
32	IoA-00 dam x IoA-10 sire	2	Male	0	4	4
0	IoA-10 dam x IoA-00 sire	1	Female	6	0	6
0	IoA-10 dam x IoA-00 sire	2	Female	6	0	6
0.125	IoA-10 dam x IoA-00 sire	1	Female	4	0	4
0.125	IoA-10 dam x IoA-00 sire	2	Female	6	0	6
0.25	IoA-10 dam x IoA-00 sire	1	Female	5	0	5
0.25	IoA-10 dam x IoA-00 sire	2	Female	5	0	5
0.5	IoA-10 dam x IoA-00 sire	1	Female	4	0	4

Dose	Parental P0 generation	Replicate	Sex	N unaffected	N affected	N total
0.5	IoA-10 dam x IoA-00 sire	2	Female	6	0	6
1	IoA-10 dam x IoA-00 sire	1	Female	5	0	5
1	IoA-10 dam x IoA-00 sire	2	Female	6	0	6
2	IoA-10 dam x IoA-00 sire	1	Female	4	1	5
2	IoA-10 dam x IoA-00 sire	2	Female	6	0	6
4	IoA-10 dam x IoA-00 sire	1	Female	5	0	5
4	IoA-10 dam x IoA-00 sire	2	Female	2	1	3
8	IoA-10 dam x IoA-00 sire	1	Female	3	2	5
8	IoA-10 dam x IoA-00 sire	2	Female	5	1	6
16	IoA-10 dam x IoA-00 sire	1	Female	2	2	4
16	IoA-10 dam x IoA-00 sire	2	Female	3	2	5
32	IoA-10 dam x IoA-00 sire	1	Female	1	2	3
32	IoA-10 dam x IoA-00 sire	2	Female	3	2	5
0	IoA-10 dam x IoA-00 sire	1	Male	5	0	5
0	IoA-10 dam x IoA-00 sire	2	Male	6	0	6
0.125	IoA-10 dam x IoA-00 sire	1	Male	5	0	5
0.125	IoA-10 dam x IoA-00 sire	2	Male	5	0	5
0.25	IoA-10 dam x IoA-00 sire	1	Male	5	0	5
0.25	IoA-10 dam x IoA-00 sire	2	Male	5	0	5
0.5	IoA-10 dam x IoA-00 sire	1	Male	6	0	6
0.5	IoA-10 dam x IoA-00 sire	2	Male	4	0	4
1	IoA-10 dam x IoA-00 sire	1	Male	6	0	6
1	IoA-10 dam x IoA-00 sire	2	Male	7	0	7
2	IoA-10 dam x IoA-00 sire	1	Male	6	0	6
2	IoA-10 dam x IoA-00 sire	2	Male	5	0	5
4	IoA-10 dam x IoA-00 sire	1	Male	6	0	6
4	IoA-10 dam x IoA-00 sire	2	Male	7	0	7
8	IoA-10 dam x IoA-00 sire	1	Male	2	3	5
8	IoA-10 dam x IoA-00 sire	2	Male	6	1	7
16	IoA-10 dam x IoA-00 sire	1	Male	3	2	5
16	IoA-10 dam x IoA-00 sire	2	Male	3	2	5
32	IoA-10 dam x IoA-00 sire	1	Male	5	2	7
32	IoA-10 dam x IoA-00 sire	2	Male	1	2	4
0	IoA-10 dam x IoA-10 sire	1	Female	4	0	4
0	IoA-10 dam x IoA-10 sire	2	Female	4	0	4
0.125	IoA-10 dam x IoA-10 sire	1	Female	5	0	5
0.125	IoA-10 dam x IoA-10 sire	2	Female	5	0	5
0.25	IoA-10 dam x IoA-10 sire	1	Female	4	0	4
0.25	IoA-10 dam x IoA-10 sire	2	Female	4	0	4
0.5	IoA-10 dam x IoA-10 sire	1	Female	5	0	5
0.5	IoA-10 dam x IoA-10 sire	2	Female	5	0	5
1	IoA-10 dam x IoA-10 sire	1	Female	4	0	4
1	IoA-10 dam x IoA-10 sire	2	Female	5	0	5
2	IoA-10 dam x IoA-10 sire	1	Female	5	0	5
2	IoA-10 dam x IoA-10 sire	2	Female	4	0	4
4	IoA-10 dam x IoA-10 sire	1	Female	5	0	5
4	IoA-10 dam x IoA-10 sire	2	Female	5	1	6
8	IoA-10 dam x IoA-10 sire	1	Female	3	2	5

Dose	Parental P0 generation	Replicate	Sex	N unaffected	N affected	N total
8	IoA-10 dam x IoA-10 sire	2	Female	5	0	5
16	IoA-10 dam x IoA-10 sire	1	Female	5	1	6
16	IoA-10 dam x IoA-10 sire	2	Female	4	2	6
32	IoA-10 dam x IoA-10 sire	1	Female	1	4	5
32	IoA-10 dam x IoA-10 sire	2	Female	3	2	5
0	IoA-10 dam x IoA-10 sire	1	Male	5	0	5
0	IoA-10 dam x IoA-10 sire	2	Male	6	1	7
0.125	IoA-10 dam x IoA-10 sire	1	Male	6	0	6
0.125	IoA-10 dam x IoA-10 sire	2	Male	5	0	5
0.25	IoA-10 dam x IoA-10 sire	1	Male	4	0	4
0.25	IoA-10 dam x IoA-10 sire	2	Male	6	0	6
0.5	IoA-10 dam x IoA-10 sire	1	Male	6	0	6
0.5	IoA-10 dam x IoA-10 sire	2	Male	6	0	6
1	IoA-10 dam x IoA-10 sire	1	Male	6	0	6
1	IoA-10 dam x IoA-10 sire	2	Male	6	0	6
2	IoA-10 dam x IoA-10 sire	1	Male	6	0	6
2	IoA-10 dam x IoA-10 sire	2	Male	7	0	7
4	IoA-10 dam x IoA-10 sire	1	Male	6	0	6
4	IoA-10 dam x IoA-10 sire	2	Male	6	0	6
8	IoA-10 dam x IoA-10 sire	1	Male	4	1	5
8	IoA-10 dam x IoA-10 sire	2	Male	5	1	6
16	IoA-10 dam x IoA-10 sire	1	Male	4	0	4
16	IoA-10 dam x IoA-10 sire	2	Male	3	2	5
32	IoA-10 dam x IoA-10 sire	1	Male	1	5	6
32	IoA-10 dam x IoA-10 sire	2	Male	3	3	6
0	IoA-02 dam x IoA-02 sire	1	Female	6	0	6
0	IoA-02 dam x IoA-02 sire	2	Female	6	0	6
1	IoA-02 dam x IoA-02 sire	1	Female	5	0	5
1	IoA-02 dam x IoA-02 sire	2	Female	4	0	4
2	IoA-02 dam x IoA-02 sire	1	Female	4	0	4
2	IoA-02 dam x IoA-02 sire	2	Female	5	0	5
4	IoA-02 dam x IoA-02 sire	1	Female	5	0	5
4	IoA-02 dam x IoA-02 sire	2	Female	5	0	5
8	IoA-02 dam x IoA-02 sire	1	Female	6	0	6
8	IoA-02 dam x IoA-02 sire	2	Female	5	0	5
16	IoA-02 dam x IoA-02 sire	1	Female	5	2	7
16	IoA-02 dam x IoA-02 sire	2	Female	4	1	5
32	IoA-02 dam x IoA-02 sire	1	Female	2	4	6
32	IoA-02 dam x IoA-02 sire	2	Female	4	2	6
0	IoA-02 dam x IoA-02 sire	1	Male	4	0	4
0	IoA-02 dam x IoA-02 sire	2	Male	5	0	5
1	IoA-02 dam x IoA-02 sire	1	Male	5	0	5
1	IoA-02 dam x IoA-02 sire	2	Male	5	0	5
2	IoA-02 dam x IoA-02 sire	1	Male	5	0	5
2	IoA-02 dam x IoA-02 sire	2	Male	5	0	5
4	IoA-02 dam x IoA-02 sire	1	Male	6	0	6
4	IoA-02 dam x IoA-02 sire	2	Male	6	0	6
8	IoA-02 dam x IoA-02 sire	1	Male	5	0	5

Dose	Parental P0 generation	Replicate	Sex	N unaffected	N affected	N total
8	IoA-02 dam x IoA-02 sire	2	Male	4	1	5
16	IoA-02 dam x IoA-02 sire	1	Male	2	3	5
16	IoA-02 dam x IoA-02 sire	2	Male	4	1	5
32	IoA-02 dam x IoA-02 sire	1	Male	3	2	5
32	IoA-02 dam x IoA-02 sire	2	Male	0	4	4

	ND1	ND5	COX1	COX3
***L. salmonis* (IoA-00)**	[...] YVISSLGGFLWVWSRV [...]	KVGSVSSLLNWGGSF [...]	FWFLMPSLSLLLMSA [...]	PLLSSFAGGFVALGLL [...]
***L. salmonis* (IoA-02)**	[...] YVISSLGSFLWVWSRV [...]	KVGSVSSSLNWGGSF [...]	FWFLMPSSSLLLMSA [...]	PLLSSFAGEFVALGLL [...]
Caligus clemensi	[...] YLISSMGCFIWVWSRV [...]	KVGNISRIFF-EKGN [...]	FWFLMPSLTLLLLSA [...]	PLIRSLAGGFITLALL [...]
Caligus rogercresseyi	[...] YIIMSCGCFVWVWSRV [...]	NSSNMGVLVLLEEDP [...]	F-FLIPSLTLLLLRA [...]	PLIRSFAGAFIATGLL [...]
Tigriopus californicus	[...] SVGSLVFVFFWMWTRA [...]	FKWSGEPLSNWSEKS [...]	FWFLMPSLLLLLLSG [...]	PLLAAAGGLFITSGML [...]
Paracyclopina nana	[...] VCMAICFSGLWIILRS [...]	SFNKGEVSFMASEDD [...]	FWFLMPALFCLLASS [...]	PLMSCLAASGIASGLL [...]
Calanus hyperboreus	[...] YLSTTGLVGVWIWART [...]	NMSKSETFFSMLEGD [...]	FWFLMPALIMLLSSS [...]	PLFGSMGGLYLTTGMV [...]
Squilla mantis	[...] CLKLVFMVFSFIWVRG [...]	GSFNMGSLHTVNDES [...]	FWLLPPALTLLLSSG [...]	PLTGSISAMMLTTGLV [...]
Tetraclita japonica	[...] -LKVGLFISLVLWLRG [...]	RDYVLGGSSNMSDGW [...]	FWLLPPALMLLISGS [...]	PLTASIGALTLTSGLS [...]
Homarus americanus	[...] YAKLVGVSFAFIWVRG [...]	GLFNLSSMSQVNDKS [...]	FWLLPFSLTLLLTSG [...]	PLTGSVSAMMLTTGLV [...]
Vargula hilgendorfii	[...] PVVLWFFGFVYLWARA [...]	QGYGKNSYVNSEELN [...]	FWLLPPSLLLLLVSS [...]	PLLTGMCVLMTVSGLI [...]
Daphnia pulex	[...] LVSFGVMAFIFVWVRG [...]	GPYGGGTSISVCESD [...]	FWFLPPALTLLLVGG [...]	PILSAFSVMSLVSGLA [...]
Artemia francisana	[...] -LMFCLVVYSYLWSRG [...]	CSYNYNLSCQYSDEE [...]	FWMLPPSLTLLLASS [...]	PLATGMGAFAMTSGLV [...]
Triops cancriformis	[...] LVKFLFIVFIFVWVRG [...]	KIPSFSCMNNFNDSD [...]	FWLLPPALTLLLSGG [...]	PLLGALGALILTTGMA [...]
	Gly251Ser	**Leu411Ser**	**Leu107Ser**	**Gly33Glu**

Figure S3.1 Partial alignment of the predicted amino acids encoded by NADH dehydrogenase subunits 1 (ND1) and 5 (ND5), and cytochrome-c-oxidase subunits 1 (COX1) and 3 (COX3) in *L. salmonis* and 13 crustacean species. Deltamethrin resistance in *L. salmonis* strains derived from field isolates has previously been shown to be associated with four non-synonymous single nucleotide polymorphisms in the mitochondrial genome, G3338A (COX3 Gly33Glu), T5889C (ND5 Leu411Ser), G8134A (ND1 Gly251Ser), and T8600C (COX1 Leu107Ser). Positions of the amino acids affected by these SNPs are boxed in the alignment. n.a.: sequence not available. NCBI accession numbers: *L. salmonis* (COX3: SFW10606.1; ND5: SFW10608.1; COX1: SFW10611.1; ND1: SFW10610.1), *C. clemensis* (COX3: HQ157566.1 translation gene 3165-3998; ND5: HQ157566.1 translation gene 4583-6256; COX1: ADM67904.1; ND1: ADM67903.1), *C. rogercresseyi* (COX3: HQ157565.1 translation gene 3177-3995; ND5: ADM67895.1; COX1: HQ157565.1 translation gene 8213-9751; ND1: ADM67897.1), *T. californicus* (COX3: ABI33097.1; ND5: ABI33098.1; COX1: ABI33091.1; ND1: ABI33093.1), *P. nana* (COX3: ACK86653.1; ND5: ACK86649.1; COX1: ACK86645.1; ND1: ACK86655.1), *C. hyperboreus* (COX3: YP_007026102.1; ND5: YP_007026108.1; COX1: YP_007026098.1; ND1: YP_007026109.1), *E. bungii* (COX3: BAD19000.1; COX1: BAD18993.1), *S. mantis* (COX3: YP_054549.1; ND5: YP_054551.1; COX1: YP_054545.2; ND1: YP_054556.2), *T. japonica* (COX3: YP_022492.1; ND5: YP_022494.1; COX1: YP_022488.1; ND1: YP_022499.1), *H. americanus* (COX3: YP_004563975.1; ND5: YP_004563977.1; COX1: YP_004563971.1; ND1: YP_004563982.1), *V. hilgendorfii* (COX3: NP_954731.1; ND5: NP_954732.1; COX1: NP_954727.1; ND1: NP_954739.1), *D. pulex* (COX3: NP_008626.1; ND5: NP_008628.1; COX1: NP_008622.1; ND1: NP_008633.1), *A. franciscana* (COX3: NP_007113.1; ND5: NP_007115.1; COX1: NP_007109.1; ND1: NP_007120.1), *T. cancriformis* (COX3: NP_775070.1; ND5: NP_775072.1; COX1: NP_775066.1; ND1: NP_775077.

Clade	Species	NCBI accession no.	Superfamily α/β hydro-lase[†] PF00561	Family[†] PF00135	Disulfide C 66	Disulfide C 93	Oxyanion hole GG 149, 150	Catalytic triad: Serine residue GXSXG[‡] 238	Serine residue S 264	Disulfide C 292	Disulfide C 307	Catalytic triad: Acidic residue E or D 367	Catalytic triad: Histidine residue H 480
J	*D. melanogaster*	DmAChE 1QO9_A	✓	✓	ATCVQE	EDCLYI	WIYGGGFM	GESAGS	MQSGT	CNCNA	MSCMR	RDEGTY	VLHGDE
E	*D. melanogaster*	NP_788501.1	✓	✓	VECMQW	EDCLTV	LLHGGAFM	GHSAGG	SVSGN	VGCGH	KDCLK	TEDGGY	TVHGDD
E	*D. melanogaster*	NP_001261749.1	✓	✓	VACLQW	EDCLTV	HIHGGAFM	GHSAGG	SFSGN	VGCES	KKCLK	TEDGGY	TVHGDD
E	*A. mellifera*	NP_001303565.1	✓	✓	PICLQR	EDCLYL	WFHGGGWQ	GESAGG	AQSGT	VGCGN	LECLR	AEEGLL	ACHAEE
E	*D. melanogaster*	NP_001011563.1	✓	✓	FPCLQY	EDCLYL	WIHGGAFQ	GLSAGG	SISGT	MGCPT	IRCLR	SEEGLY	VCHADD
E	*A. mellifera*	NP_001119716.1	✓	✓	SVCMQY	EDCLYI	WIHGGAFQ	GMSAGG	SISGV	MKCRT	IDCLQ	SKEGLY	VCHGDD
E	*A. mellifera*	XP_006566930.1	✓	✓	NICVQR	EDCLYL	WFHGCGWI	GESAGG	SQSGN	LGCPS	VDCLR	SQEGSL	VSHADE
E	***L. salmonis***	**HACA01030908.1[§]**	✓	✓	**HFCPQH**	**EDCLWL**	**WIHGGNFV**	**GQQAGG**	**SLSGS**	**LECPY**	**IECIR**	**DDEGAF**	**VGNGDD**

Figure S4.1 Conserved carboxylesterase (CaE) motifs in *Apis mellifera*, *Drosophila melanogaster*, and *Lepeophtheirus salmonis* CaE sequences from clade E. CaE sequences were aligned against the reference *Drosophila melanogaster* acetylcholine esterase (DmAChE) sequence. Amino acid residues were numbered according to DmAChE. Conserved catalytic triad residues (Ser238, Glu/Asp367, and His480) are shown in green. Additional conserved amino acid residues within the active site (oxyanion hole G149 and G150, putative catalytic tetrad residue Ser264 (Thomas et al., 1999)) are shown in blue. Conserved disulphide bridges (Cys66, Cys98 and Cys292, Cys307) are shown in yellow. "-" indicates a gap in the alignment. [†]Typology according to Pfam (PF) entries. [‡]Nucleophilic elbow. [§]RT-PCR followed by Sanger sequencing was used to confirm cDNA sequences, which were deposited in the European Nucleotide Archive (see Table S4.4 for accession numbers).

P1 subsite (leaving pocket)

P2 subsite (acyl pocket)

Oxyanion hole

Catalytic triad

Clade	NCBI accession no.	Changed AA within CaE gene	Gly, Gly	variable residues	Ser	variable residues	variable residues	variable residues	Glu or Asp	variable residues	variable residues	variable residues	His
J	**DmAChE 1QO9_A**		**149, 150**	**162**	**238**	**271**	**328**	**330**	**367**	**370**	**371**	**440**	**480**
O	**HACA01008519.1**	P **95** P L **374** V L **375** Q E **581** *	132, 133	143	212	247	300	302	344	347	348	423	466
O	**HACA01024270.1**	S **148** R F **149** S Q **150** * Q **150** R S **152** P	108, 109	121	190	224	279	281	326	429	430	407	472
1A	**HACA01023258.1**	F **362** Y K **431** Q	148, 149	161	230	263	319	321	358	361	362	432	472
1B	**HACA01002875.1**	I **88** M S **568** P V **579** E	148, 149	161	230	263	319	321	358	361	362	432	472
H	**HACA01028197.1**	A **338** A	254, 255	265	334	369	-	421	476	479	480	559	610

Figure S4.2 Positions of mutated amino acids (AA) based on data shown in Table S4.8 and active site residues within carboxylesterase (CaE) genes. L. salmonis CaE sequences were aligned against the reference Drosophila melanogaster acetylcholine esterase (DmAChE) sequence. Amino acid residues were numbered according to DmAChE. Catalytic triad residues are shown in green, residues of the oxyanion hole are shown in grey, residues of the P1 subsite (leaving pocket) are shown in blue, and residues of the P2 (acyl pocket) are shown in orange. Residues of the catalytic triad and the oxyanion hole show strong conservation across CaEs, while those constituting the P1 and P2 subsites are more variable. "-" indicates a gap in the alignment. "*" indicates a stop codon.

Table S4.1 The carboxylesterase (CaE) family in *L. salmonis*. CaEs were identified by homology searches in transcriptome (EBI ENA reference ERS237607) and genome assemblies (LSalAlt2s, ensemble.metazoa.org), using the entire complement of *Drosophila melanogaster* as queries. The assignment of sequences to classes and clades is based on the phylogenetic analysis shown in Fig. 4.1.

Annotation									
Class	**Clade**	**Transcript annotation**	**Transcript accession no. (NCBI)**	**Length (aa)**	**Gene accession no. (EnsemblMetazoa)**	**Best BLAST hit in *D. melanogaster***	**NCBI accession number**	**E-value**	**Identity (%)**
2	**E**	Venom carboxylesterase 6 like	HACA01030908.1[†,§,‡]	647	EMLSAG00000000224	uncharacterized protein, isoform B (CG6414)	NP_001303565.1	2.00E-84	34.8
	H	Putative protein	HACA01028197.1[§,‡]	742	EMLSAG00000010508	uncharacterized protein (CG9287)	NP_609244.1	5.00E-40	28.35
	H	Hypothetical protein	HACA01016812.1[§,‡]	695	EMLSAG00000010605	alpha esterase	AAB01153.1	8.00E-38	27.62
	O	Hypothetical protein	HACA01024270.1[§,‡]	583	EMLSAG00000003802	uncharacterized protein, isoform B (CG6414)	NP_001303565.1	8.00E-55	28.95
	O	Putative esterase like	HACA01010127.1[†,§,‡]	302	EMLSAG00000004652	alpha-esterase	AAB01142.1	0.009	17.73
	O	Putative protein	HACA01001173.1[§,‡]	587	EMLSAG00000008692	uncharacterized protein, isoform B (CG6414)	NP_001303565.1	3.00E-63	31.78
	O	Venom carboxylesterase 6 like	HACA01028341.1[†,§,‡]	410	EMLSAG00000011806	uncharacterized protein, isoform B (G10175)	NP_732875.1	1.00E-28	27.96
	O	Juvenile hormone esterase like [*Tribolium castaneum*]	HACA01008519.1[†,§,‡]	589	EMLSAG00000011990	uncharacterized protein (CG4382)	NP_609301.2	7.00E-64	33.86
3	**J**	Acetylcholinesterase 1B	HACA01002875.1[§]	583	EMLSAG00000002841	acetylcholine esterase, isoform C (CG17907)	NP_001262530.1	4.00E-128	36.25
	J	Acetylcholinesterase 1A	HACA01023258.1[§]	630	EMLSAG00000002842	acetylcholine esterase, isoform C (CG17907)	NP_001262530.1	1.00E-129	36.71
	I	CG4382 PA like [*Tribolium castaneum*]	HACA01002103.1[§]	523	EMLSAG00000004103	uncharacterized protein (CG4382)	NP_609301.2	1.00E-42	28.04
	I	Esterase FE4 like [*Apis mellifera*]	HACA01023586.1[§]	556	EMLSAG00000008264	uncharacterized protein (CG3841)	NP_001188759.1	6.00E-74	33.93
	K	Gliotactin [*Drosophila melanogaster*]	HACA01010572.1[†,§]	611	EMLSAG00000008163	gliotactin, isoform A (CG3903)	NP_476602.1	0.00E+00	57.2
	L	Hypothetical protein	HACA01030603.1[†,#,§]	697	EMLSAG00000000872	neuroligin 3, isoform B (CG34127)	NP_001036685.2	3.00E-43	28.71
	L	Putative protein	HACA01001096.1	744	EMLSAG00000007250[+,†,§]	neuroligin 3,	NP_001036685.2	2.00E-94	33.04
	L	Putative protein	HACA01001097.1			isoform B (CG34127)			
	L	Neuroligin 4	HACA01001453.1	761	EMLSAG00000001202[++,§]	neuoligin 1,	NP_001246966.1	1.00E-57	33.18
	L	Neuroligin 4	HACA01024283.1			isoform E (CG31146)			

Annotation									
Class	**Clade**	**Transcript annotation**	**Transcript accession no. (NCBI)**	**Length (aa)**	**Gene accession no. (EnsemblMetazoa)**	**Best BLAST hit in *D. melanogaster***	**NCBI accession number**	**E-value**	**Identity (%)**
	L	Putative protein	HACA01025815.1	772	EMLSAG00000001229*,§	neuroligin 3, isoform B (CG34127)	NP_001036685.2	3.00E-60	33.82
	L	Neuroligin 1 like	HACA01027426.1§						
	L	Putative protein	HACA01030315.1§						
	L	Neuroligin 4	HACA01004593.1§						
	L	Hypothetical protein	HACA01005582.1†,§	138	EMLSAG00000001231	neuroligin 3, isoform B (CG34127)	NP_001036685.2	2.00E-34	49.56
	L	-	-	147	EMLSAG00000007248†,§	neuroligin 4, isoform C (CG34139)	NP_001036730.2	1.00E-25	57.14
	M	Neurotactin like [*Tribolium castaneum*]	HACA01032517.1§	401	EMLSAG00000003706	neurotactin, isoform C (CG9704)	NP_001189121.1	6.00E-65	35.41
	M	Putative protein	HACA01011916.1§	648	EMLSAG00000010413	esterase 6, isoform B (CG6917)	NP_001261749.1	6.00E-38	35.54

⁺Gene model EMLSAT00000007250 is the fusion between two transcript models, probably reflecting an assembly problem.

⁺⁺Gene model EMLSAT00000001202 is the fusion between two transcript models, probably reflecting an assembly problem.

*Gene model EMLSAT00000001229 is the fusion between four transcript models, probably reflecting an assembly problem.

†Partial sequence only.

#Alternative splicing forms exist.

§Predicted polypeptide length based on this sequence.

‡RT-PCR followed by Sanger sequencing was used to confirm cDNA sequences, which were deposited in the European Nucleotide Archive (see Table S4.4 for accession numbers).

Table S4.2 The *Apis mellifera* and *Drospophila melanogaster* carboxylesterase (CaE) family.

Class	Clade	Species	NCBI accession no.	NCBI annotation	Superfamily α/β hydrolase fold[†] PF00561/IPR029058	Family CaE Type B[†] PF00135/IPR002018	Serine active site[†] PS00122/IPR19826
1	**A**	*Apis mellifera*	XP_392698.3	esterase FE4	✓	✓	✓
	A	*Apis mellifera*	XP_026299635.1	bile salt-activated lipase isoform X2	✓	✓	✓
	A	*Apis mellifera*	NP_001128419.1	esterase A2	✓	✓	✓
	A	*Apis mellifera*	XP_006564307.1	carboxylesterase isoform X1	✓	✓	✓
	A	*Apis mellifera*	XP_026297794.1	esterase FE4	✓	✓	✓
	A	*Apis mellifera*	XP_016770320.2	esterase B1	✓	✓	✓
	B	*Drosophila melanogaster*	NP_536784.1	cricklet	✓	✓	
	B	*Drosophila melanogaster*	NP_001287487.1	uncharacterized protein	✓	✓	✓
	C	*Drosophila melanogaster*	NP_524266.1	alpha-Esterase-4	✓	✓	
	C	*Drosophila melanogaster*	NP_001287210.1	alpha-Esterase-3, isoform D	✓	✓	
	C	*Drosophila melanogaster*	NP_524262.1	alpha-Esterase-6	✓	✓	
	C	*Drosophila melanogaster*	NP_001246965.1	alpha-Esterase-5, isoform B	✓	✓	
	C	*Drosophila melanogaster*	NP_001262345.1	alpha-Esterase-2, isoform B	✓	✓	
	C	*Drosophila melanogaster*	NP_611678.1	gasoline	✓	✓	
	C	*Drosophila melanogaster*	NP_524269.3	alpha-Esterase-1	✓	✓	
	C	*Drosophila melanogaster*	NP_524261.1	alpha-Esterase-7	✓	✓	✓
	C	*Drosophila melanogaster*	NP_001246963.1	alpha-Esterase-10, isoform D	✓	✓	✓
	C	*Drosophila melanogaster*	NP_524259.2	alpha-Esterase-8	✓	✓	✓
	C	*Drosophila melanogaster*	NP_731165.2	alpha-Esterase-9, isoform D	✓	✓	✓
2	**D**	*Drosophila melanogaster*	NP_001188759.1	uncharacterized protein	✓	✓	✓
	D	*Drosophila melanogaster*	NP_609301.2	uncharacterized protein	✓	✓	
	D	*Drosophila melanogaster*	NP_001262469.1	uncharacterized protein	✓	✓	✓
	D	*Apis mellifera*	XP_393293.2	esterase E4	✓	✓	✓
	E	*Drosophila melanogaster*	NP_788501.1	esterase P	✓	✓	✓
	E	*Drosophila melanogaster*	NP_001261749.1	esterase 6, isoform B	✓	✓	✓
	E	*Drosophila melanogaster*	NP_001303565.1	uncharacterized protein	✓	✓	✓
	E	*Apis mellifera*	NP_001011563.1	juvenile hormone esterase precursor	✓	✓	✓
	E	*Apis mellifera*	NP_001119716.1	venom carboxylesterase-6 precursor	✓	✓	✓
	E	*Apis mellifera*	XP_006566930.1	venom carboxylesterase-6-like	✓	✓	✓
	F	*Apis mellifera*	XP_006568129.2	venom carboxylesterase-6-like	✓	✓	

Class	Clade	Species	NCBI accession no.	NCBI annotation	Superfamily α/β hydrolase fold[†] PF00561/IPR029058	Family CaE Type B[†] PF00135/IPR002018	Serine active site[†] PS00122/IPR19826
	G	*Drosophila melanogaster*	NP_611085.2	juvenile hormone esterase duplication	✓	✓	✓
	G	*Drosophila melanogaster*	NP_001286476.1	juvenile hormone esterase, isoform C	✓	✓	✓
	H	*Drosophila melanogaster*	NP_649321.1	esterase Q	✓	✓	
	H	*Drosophila melanogaster*	NP_001245946.1	glutactin, isoform D	✓	✓	
	H	*Drosophila melanogaster*	NP_609244.1	uncharacterized protein	✓	✓	
	H	*Drosophila melanogaster*	NP_609245.1	uncharacterized protein	✓	✓	
3	**J**	*Drosophila melanogaster*	NP_001262530.1	acetylcholine esterase, isoform C	✓	✓	✓
	J	*Apis mellifera*	NP_001035320.1	acetylcholinesterase 2 precursor	✓	✓	✓
	J	*Apis mellifera*	XP_006570397.2	acetylcholinesterase	✓	✓	✓
	I	*Drosophila melanogaster*	NP_001259865.1	uncharacterized protein	✓	✓	
	I	*Drosophila melanogaster*	NP_611881.1	uncharacterized protein	✓	✓	
	I	*Apis mellifera*	XP_393670.5	esterase E4	✓	✓	
	I	*Apis mellifera*	XP_016768436.1	carboxylesterase 5A	✓	✓	✓
	K	*Drosophila melanogaster*	NP_723931.2	gliotactin, isoform F	✓	✓	
	K	*Apis mellifera*	XP_016770618.2	neuroligin-4, Y-linked isoform X3	✓	✓	
	L	*Drosophila melanogaster*	NP_001246966.1	neuroligin 1, isoform E	✓	✓	✓
	L	*Drosophila melanogaster*	NP_001285693.1	neuroligin 2, isoform C	✓	✓	
	L	*Drosophila melanogaster*	NP_731170.2	neuroligin 3, isoform C	✓	✓	
	L	*Drosophila melanogaster*	NP_001287420.1	neuroligin 4, isoform E	✓	✓	
	L	*Apis mellifera*	XP_006561900.2	uncharacterized protein	✓	✓	
	L	*Apis mellifera*	XP_016773176.2	neuroligin-1 isoform X3	✓	✓	
	L	*Apis mellifera*	XP_016769339.1	neuroligin 5 isoform X1	✓	✓	
	L	*Apis mellifera*	NP_001139208.1	neuroligin 3 precursor	✓	✓	
	L	*Apis mellifera*	NP_001139209.1	neuroligin 4 precursor	✓	✓	
	M	*Drosophila melanogaster*	NP_001189121.1	neurotactin, isoform C	✓	✓	
	M	*Drosophila melanogaster*	NP_001286421.1	uncharacterized protein	✓	✓	
	M	*Apis mellifera*	XP_016771802.2	neurotactin	✓	✓	

[†]Typology according to Pfam (PF), InterPro (IPR), and Prosite (PS) entries.

Table S4.3 Oligonucleotide primer sequences used in quantitative reverse transcription polymerase chain reaction (RT-qPCR analyses).

Clade/Transcript annotation	NCBI accession no.	Forward primer (5' - 3')	Reverse primer (5' - 3')	Amplicon size (nt)	Efficacy (%)	Annealing tempera-ture (°C)
Target genes						
E	HACA01030908.1	TCGAAGTAGACCAGATACGGAG	TTGGGCTCAGCATTTTCAGC	102	86	60
J, acetylcholinesterase 1B	HACA01002875.1[†]	CATTGTCATGGAGTCTGGGATC	GGGAGGTTGTGCGTATGGTA	144	87	60
J, acetylcholinesterase 1A	HACA01023258.1[‡]	ACGCCAATTATTCTCGAGTTCTC	AGAACGTGGATGCAAAGGAC	196	97	60
H	HACA01028197.1	AGATGTTGGGAAGTACGGGG	CGAAATTAAAGTCGCAGCAAGA	141	86	60
H	HACA01016812.1	CGCACAATGGAGATACGATCA	CCATAGGGTTTCGGCAATTTC	125	88	60
O	HACA01024270.1	CCTTCTTGTACCATGGATTCCC	TCTTCGTATCGTTTCCTCCAAG	145	82	60
O	HACA01010127.1	GGAGGGGATGTCTTTTCTCTTTC	GACTGATGCCTGGACGTTTG	104	96	60
O	HACA01001173.1	ACCTGATTTTCCATCTCAAGTGC	GGAGAGAAACCCATCGTGATC	149	84	60
O	HACA01028341.1	GGGGTTGAGGATTGTCTTGTC	AGATCCACTCAACAATGCGTAG	118	89	60
O	HACA01008519.1	TGCTAACTCTTCTCATGGTCC	GAAGCACCAAGATCCAGTCC	94	84	60
Reference genes						
Ribosomal subunit 40S	BT121430.1	AGTGTGGCCGGTGTTTAACAATCATCAA	GGGCTTCGAGTCCTTGTATGCTGCTGCT ACT	86	94	60
Ribosomal subunit 60S	ACO10279.1	CCTAGCTGCAATCACCATGA	CTCTTGCACTTGCTGCACTC	197	87	55
Elongation factor 1-alpha	EF490880.1	CCAAATTAAGGAAAAGGTCGACAGACGTAC TG	CAATGCCGGCATCACCAGACTTGA	86	96	60
Hypoxanthine-guanine phosphoribosyltransferase	ACO14905.1	GCAGCAAACATCGAATCTCA	TCTTTGCACGAACAAACTGC	187	91	55
RMD-5 homologue	ACO15319.1	TCTCCTTATGCCCACTTGCT	GAGTTCCGTCCTTTGCATTC	220	93	55

[†]Corresponding to NCBI nucleotide KJ132369.1.

[‡]Corresponding to NCBI nucleotide KJ132368.1.

Table S4.4 Oligonucleotide primer sequences used in rapid amplification of 3' and 5' cDNA ends (RACE) and subsequent sequencing of *L. salmonis* cDNA sequence.

Clade	NCBI accession no.	Gen specific primer 3' RACE (5' - 3')	Gen specific primer 5' RACE (5' - 3')	Forward primer	Reverse primer	Additional sequencing primer 1	Additional sequencing primer 2	Additional sequencing primer 3	RT-PCR product sequence ENA accession number
E	HACA01030908.1	TGACGAAGGTGCTTTCAAAGGCTCTGC	GCCAAGTCGCGGGAAAATACATTGAGCC	GAACAAACGCAGATAAACACATC	TCATCCTTATCTTGAAGAGGTGG	AAAGCCCCTGTTCCCTTC	CCCTGGAGCGTGCAAAG	–	LR898355
H	HACA01028197.1	AGATGTTGGGAAGTACGGGGCATGCC	GTCCGTGACGTGAATAGGCCCATAATGT	GGGGAAAGTATGTGGGTTCG	TGACTGTGACTCCGCTTCTC	GAAACAACACGAGCCTGG	TGAAAGTTGGGAGTTGGC	–	LR898354
H	HACA01016812.1	TATATGATGTCCAAGCACGCCAGC	GCCAAGATCAAAGTGCTTCCTCCTTTCC	TCGCACAATGGAGATACGATC	AGCAGCAACATTCAAGGAAGG	GTTGGATGTGGACTTTGGC	TCCATGGGCCTTTGACAG	GGACGCAAATATTGAAAGCC	LR898353
O	HACA01024270.1	CCATGGAAGCCCTGTGTTGATGGTGG	GGAAGTACCGCCAAAGTGATGTATGTGG	GAGCTTGACCTTGGCCAATC	TTGTATTTCGAGCTTTCATCCAC	GGTGGAGCATTCATTCTTGG	TCTTACTCGCGTCTTGTCC	ATGGCTTTCAAGTGTACGG	LR898351
O	HACA01010127.1	CTGTTCATGGTGGATCGGCTCAGC	CCCCTAGAACAGTCCAATCCCTTCGTA	GTCGAGCAAGGGAAACATCC	GGCGAGGAGGAAACGGATAG	TGATGGGCCATGATGCAG	AACGTCACGACACTCTGG	–	LR898352
O	HACA01001173.1	TGGATTGCGGAAATGGGATGTATCGGTG	GACTCATTGGATAGCTGCAGGGACGC	CATGGGCAAAGATGTCTGAGA	GGTGATATCCGTGCTCAGTC	GAGAAGAGTACGCGAGGG	GGCAACATCACGCTTCATG	–	LR898356
O	HACA01028341.1	CCCAAGGGCCAACTATCCTACAGTCAAA	TTGACTGTAGGATAGTTGGCCCTTGGGT	ACCAAAGGAGATGTTCGTGG	AATTTCACCGGCTTCGTCTG	TTCTGGTGCTCATTCCGC	CCCAAGGGCCAACTATCC	–	LR898349
O	HACA01008519.1	ACAGATGTTCACTTCCTCGGACCCATC	ACATCTGCCTCATCGATTCCCATCAGG	ACAGTGTTTCTTGTAGTAGAGGA	CGACCTCTCTCTCCATGACAG	TCCCAAAGAAAGGAAGGTTC	GCAATGAGATGGGATGTGAC	GTTCACTTCCTCGGACCC	LR898350

Table S4.5 Predicted subcellular localization and signal peptides of *L. salmonis* carboxylesterase sequences. Likelihood probability of subcellular localization predicted by DeepLoc-1.0, while signalP version 5.0 was used to predict putative signal peptide sequences.

Class	Clade	Identifier	Type† (Likelihood probability)	Signal peptide secretory pathway Likelihood probability	Localisation (Likelihood probability)
2	**H**	HACA01028197.1‡,+	soluble (0.67)	0.248	Lysosome (0.427)
	H	HACA01016812.1‡,+	soluble (0.947)	0.953	Endoplasmic reticulum (0.674)
	O	HACA01024270.1‡,+	soluble (0.883)	0.002	Cytoplasm (0.371)
	O	HACA01001173.1‡,+	soluble (0.952)	0.069	Endoplasmic reticulum (0.593)
	O	HACA01008519.1‡,+	soluble (0.969)	0.967	Endoplasmic reticulum (0.746)
	O	HACA01010127.1‡,+	soluble (0.916)	0.0004	Cytoplasm (0.682)
	O	HACA01028341.1‡,+	soluble (0.698)	0.001	Cytoplasm (0.375)
	E	HACA01030908.1‡,+	soluble (0.868)	0.619	Extracellular (0.347)
3	**J**	HACA01002875.1‡	soluble (0.864)	0.325	Endoplasmic reticulum (0.477)
	J	HACA01023258.1‡	membrane (0.98)	0.407	Cell membrane (0.559)
	I	HACA01002103.1‡	soluble (0.998)	0.997	Extracellular (0.894)
	I	HACA01023586.1‡	soluble (0.965)	0.988	Endoplasmic reticulum (0.909)
	K	HACA01010572.1‡	membrane (1)	0.011	Cell membrane (0.998)
	L	HACA01030603.1‡	membrane (1)	0.001	Cell membrane (0.952)
	L	EMLSAG00000007248§	membrane (0.127)	0.001	Nucleus (0.132)
	L	EMLSAG00000007250§	membrane (1)	0.005	Cell membrane (0.973)
	L	EMLSAG00000001202§	membrane (1)	0.011	Cell membrane (0.935)
	L	EMLSAG00000001229§	membrane (1)	0.004	Cell membrane (0.999)
	L	HACA01005582.1‡	membrane (0.281)	0.001	Mitochondrium (0.434)
	M	HACA01032517.1‡	soluble (0.886)	0.001	Cytoplasm (0.252)
	M	HACA01011916.1‡	soluble (0.963)	0.547	Endoplasmic reticulum (0.455)

†Proteins were classified as membrane or soluble if they were found on either the membrane or the lumen of the organelle.

‡NCBI Nucleotide accession number.

§EnsemblMetazoa accession number.

+RT-PCR followed by Sanger sequencing was used to confirm cDNA sequences, which were deposited in the European Nucleotide Archive (see Table S4.4 for accession numbers).

Table S4.6 Cycle threshold (Ct) values and estimated relative copy numbers of the three most stable reference genes (ribosomal subunit 40S, 40S; ribosomal subunit 60S, 60S; elongation factor 1-alpha, efa) and ten carboxylesterase transcripts in *L. salmonis*.

Strain	Sex	Drug	Dose	Av. Ct value 40s	Av. Ct value 60s	Av. Ct value efa	Relat. copy no. 40s	Relat. copy no. 60s	Relat. copy no. efa
IoA-00	**F**	**PEG300**	**0**	17.44	18.63	16.6	60.56475	63.19562	65.95045967
IoA-00	**F**	**PEG300**	**0**	17.94	18.71	16.835	43.3489	59.84676	56.33028832
IoA-00	**F**	**PEG300**	**0**	17.57	18.84	16.7	55.53109	55.17804	61.69315917
IoA-00	**F**	**PEG300**	**0**	17.395	18.825	16.57	62.39057	55.33377	67.1700057
IoA-00	**F**	**PEG300**	**0**	17.725	18.9	16.815	50.08122	52.52925	57.08285193
IoA-00	**F**	**PEG300**	**0**	17.35	18.545	16.61	64.40612	67.03839	65.48979176
IoA-00	**M**	**PEG300**	**0**	18.575	19.84	17.975	28.31762	27.57509	26.46840767
IoA-00	**M**	**PEG300**	**0**	18.585	19.615	18.05	28.12745	32.17847	25.16108328
IoA-00	**M**	**PEG300**	**0**	18.755	20.11	18.045	25.12582	22.9155	25.23706255
IoA-00	**M**	**PEG300**	**0**	18.785	19.615	17.72	24.88257	32.17092	31.35370611
IoA-00	**M**	**PEG300**	**0**	18.96	19.81	18.09	21.9075	28.17762	24.5128109
IoA-00	**M**	**PEG300**	**0**	18.485	19.85	17.82	30.14876	27.4363	29.30198452
IoA-00	**F**	**DTM**	**0.05**	17.77	18.595	16.875	48.62214	64.82586	54.85479503
IoA-00	**F**	**DTM**	**0.05**	17.375	18.6	16.56	63.23991	64.50073	67.60529979
IoA-00	**F**	**DTM**	**0.05**	17.49	18.495	16.335	58.55952	69.30688	78.68029606
IoA-00	**F**	**DTM**	**0.05**	17.58	18.575	16.6	55.11741	65.64033	65.84606888
IoA-00	**F**	**DTM**	**0.05**	17.855	18.65	16.97	45.90756	62.41589	51.54181237
IoA-00	**F**	**DTM**	**0.05**	17.56	18.24	16.48	56.22101	82.54817	71.36648096
IoA-00	**M**	**DTM**	**0.05**	18.465	19.2	17.62	30.47994	42.8008	33.58223321
IoA-00	**M**	**DTM**	**0.05**	18.265	18.965	17.305	34.86167	50.22452	41.31769934
IoA-00	**M**	**DTM**	**0.05**	18.435	19.16	17.665	31.09957	44.00575	32.64528714
IoA-00	**M**	**DTM**	**0.05**	18.375	18.595	17.505	32.3943	64.71642	36.19846819
IoA-00	**M**	**DTM**	**0.05**	18.375	18.95	17.675	32.40301	50.91245	32.28008761
IoA-00	**M**	**DTM**	**0.05**	18.38	18.945	17.595	32.28227	51.46858	34.04898241
IoA-00	**F**	**EMB**	**25**	17.44	18.4	16.535	60.57967	73.97037	68.76530672
IoA-00	**F**	**EMB**	**25**	17.385	18.27	16.675	62.82928	81.01466	62.66478632
IoA-00	**F**	**EMB**	**25**	17.35	18.435	16.16	64.31247	72.2194	88.1720635
IoA-00	**F**	**EMB**	**25**	17.52	18.15	16.27	57.4069	87.79354	82.09537508

Strain	Sex	Drug	Dose	Av. Ct value 40s	Av. Ct value 60s	Av. Ct value efa	Relat. copy no. 40s	Relat. copy no. 60s	Relat. copy no. efa
IoA-00	**F**	**EMB**	**25**	19.86	21.23	19.02	12.04965	10.64842	13.22835404
IoA-00	**F**	**EMB**	**25**	17.255	18.59	16.2	68.57515	64.96256	85.86251934
IoA-00	**M**	**EMB**	**25**	18.09	18.795	17.35	39.1798	56.43013	40.07902291
IoA-00	**M**	**EMB**	**25**	18.26	18.975	17.65	35.00106	49.96352	32.80470918
IoA-00	**M**	**EMB**	**25**	18.16	18.76	17.215	37.39056	57.86344	43.77557786
IoA-00	**M**	**EMB**	**25**	17.79	18.48	17.18	47.90581	70.02422	44.81081863
IoA-00	**M**	**EMB**	**25**	18.92	19.28	17.875	22.84538	40.47185	28.24951759
IoA-00	**M**	**EMB**	**25**	18.37	19.105	17.515	32.48089	45.64057	35.90532748
IoA-02	**F**	**PEG300**	**0**	17.78	18.18	16.56	55.95341	55.86706	53.33883375
IoA-02	**F**	**PEG300**	**0**	17.88	18.545	16.82	52.27064	44.40865	45.53254877
IoA-02	**F**	**PEG300**	**0**	17.42	17.83	16.32	70.39476	69.4625	62.9094846
IoA-02	**F**	**PEG300**	**0**	17.275	17.74	16.215	77.34616	73.28235	67.75773794
IoA-02	**F**	**PEG300**	**0**	17.475	18.26	16.13	67.93709	53.10859	71.54831437
IoA-02	**F**	**PEG300**	**0**	17.395	18.055	16.41	71.99913	60.23276	59.3231663
IoA-02	**M**	**PEG300**	**0**	18.12	18.64	17.11	44.75362	41.85018	36.62759107
IoA-02	**M**	**PEG300**	**0**	19.76	20.13	18.845	15.37768	16.55461	11.22795636
IoA-02	**M**	**PEG300**	**0**	18.225	18.99	17.155	41.69258	33.65586	35.5278785
IoA-02	**M**	**PEG300**	**0**	18.785	19.455	17.855	29.11172	25.19727	22.08356898
IoA-02	**M**	**PEG300**	**0**	18.96	19.21	17.71	25.86182	29.35238	24.36518017
IoA-02	**M**	**PEG300**	**0**	18.625	19.02	17.44	32.15497	33.03335	29.34852915
IoA-02	**F**	**DTM**	**0.05**	17.185	17.705	16.005	82.27648	74.91671	77.93898582
IoA-02	**F**	**DTM**	**0.05**	17.125	17.7	15.735	85.4318	75.11927	93.72013332
IoA-02	**F**	**DTM**	**0.05**	17.14	17.97	16.35	84.45714	63.52035	61.56411556
IoA-02	**F**	**DTM**	**0.05**	17.365	17.85	16.51	72.99149	68.41377	55.47747572
IoA-02	**F**	**DTM**	**0.05**	17.43	17.85	16.215	69.93096	68.63645	67.53066489
IoA-02	**F**	**DTM**	**0.05**	17.28	17.565	16.26	77.17127	81.69862	65.72562259
IoA-02	**M**	**DTM**	**0.05**	18.285	18.63	17.09	40.4893	42.22155	37.20090873
IoA-02	**M**	**DTM**	**0.05**	18.125	18.605	17.055	44.5059	42.81086	38.0295537
IoA-02	**M**	**DTM**	**0.05**	17.75	18.22	16.69	56.84435	54.35141	48.81906728
IoA-02	**M**	**DTM**	**0.05**	17.98	18.555	16.91	49.02033	44.15194	42.13117304

Strain	Sex	Drug	Dose	Av. Ct value 40s	Av. Ct value 60s	Av. Ct value efa	Relat. copy no. 40s	Relat. copy no. 60s	Relat. copy no. efa
IoA-02	**M**	**DTM**	**0.05**	17.725	18.32	16.785	57.82665	51.07211	45.75542972
IoA-02	**M**	**DTM**	**0.05**	17.865	18.195	16.93	52.92735	55.27124	42.07535703
IoA-02	**F**	**EMB**	**25**	17.425	17.935	16.255	70.15617	65.0011	66.33668974
IoA-02	**F**	**EMB**	**25**	17.5	18.005	16.465	66.81407	62.15808	57.19009075
IoA-02	**F**	**EMB**	**25**	17.19	17.55	16.16	81.91198	82.46124	70.3324069
IoA-02	**F**	**EMB**	**25**	17.305	17.48	16.13	75.85792	86.12784	71.75049092
IoA-02	**F**	**EMB**	**25**	17.23	17.59	16.17	79.72233	80.46659	69.98531146
IoA-02	**F**	**EMB**	**25**	17.315	17.49	16.01	75.38842	85.75765	77.66365983
IoA-02	**M**	**EMB**	**25**	18.275	18.455	17.06	40.36357	46.9669	37.90759612
IoA-02	**M**	**EMB**	**25**	18.35	18.53	17.255	38.43748	44.82926	33.22794845
IoA-02	**M**	**EMB**	**25**	18.035	18.47	17.115	47.18307	46.51588	36.68110815
IoA-02	**M**	**EMB**	**25**	18.165	18.62	17.145	43.49502	42.38006	35.8533014
IoA-02	**M**	**EMB**	**25**	18.025	18.17	16.955	47.59549	56.06377	40.86714786
IoA-02	**M**	**EMB**	**25**	17.96	18.37	16.99	49.57308	49.49879	39.83154381
IoA-02	**F**	**DTM**	**2**	17.45	17.825	16.24	59.45706	67.27042	61.6350767
IoA-02	**F**	**DTM**	**2**	17.73	17.985	16.135	49.21775	60.90425	66.12637743
IoA-02	**F**	**DTM**	**2**	17.31	17.755	16.13	65.37439	70.14748	66.44070521
IoA-02	**F**	**DTM**	**2**	17.1	17.79	15.93	75.34949	68.63341	75.87990913
IoA-02	**F**	**DTM**	**2**	17.38	18.575	16.115	62.35622	43.40498	67.01066982
IoA-02	**F**	**DTM**	**2**	17.28	18.155	16.25	66.68788	54.87219	61.17712716
IoA-02	**M**	**DTM**	**2**	18.315	18.59	17.22	33.15508	41.92888	31.84289603
IoA-02	**M**	**DTM**	**2**	18.265	18.685	17.24	34.31548	39.50775	31.4112968
IoA-02	**M**	**DTM**	**2**	18.475	19.295	17.33	29.83305	27.15736	29.58055144
IoA-02	**M**	**DTM**	**2**	18.035	18.255	17.1	40.09198	51.51072	34.52234418
IoA-02	**M**	**DTM**	**2**	18.215	18.605	17.42	35.47442	41.51574	27.82559402
IoA-02	**M**	**DTM**	**2**	18.47	19.025	17.51	29.88338	32.02797	26.19908513
IoA-02	**F**	**EMB**	**150**	17.36	17.58	16.53	63.20405	78.33429	50.66153712
IoA-02	**F**	**EMB**	**150**	17.445	18.15	16.54	59.65984	54.94835	50.3774895
IoA-02	**F**	**EMB**	**150**	17.39	17.9	16.18	61.91965	64.42961	64.33294253
IoA-02	**F**	**EMB**	**150**	17.51	18.215	16.435	57.10023	52.80653	54.07662535

Strain	Sex	Drug	Dose	Av. Ct value 40s	Av. Ct value 60s	Av. Ct value efa	Relat. copy no. 40s	Relat. copy no. 60s	Relat. copy no. efa
IoA-02	**F**	**EMB**	**150**	17.375	17.97	16.485	62.60455	61.4998	52.32677815
IoA-02	**F**	**EMB**	**150**	17.22	17.575	16.125	69.45945	78.46924	66.67870448
IoA-02	**M**	**EMB**	**150**	18.19	18.445	17.225	36.08061	45.81687	31.79433357
IoA-02	**M**	**EMB**	**150**	17.78	18.295	16.93	47.6185	50.24479	38.75689306
IoA-02	**M**	**EMB**	**150**	17.87	18.62	16.98	45.18297	41.12897	37.44082822
IoA-02	**M**	**EMB**	**150**	18.055	18.71	16.785	40.93006	39.74741	42.6890266
IoA-02	**M**	**EMB**	**150**	18.11	18.525	17.265	38.09716	43.59421	30.90749223
IoA-02	**M**	**EMB**	**150**	17.78	18.285	16.925	47.66733	50.55591	38.83674319

Table S4.6 continued: Cycle threshold (Ct) values and estimated relative copy numbers of the three most stable reference genes (ribosomal subunit 40S, 40S; ribosomal subunit 60S, 60S; elongation factor 1-alpha, efa) and ten carboxylesterase transcripts in *L. salmonis*.

Strain	Sex	Drug	Dose	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.
				HACA 01002875.1		HACA 01023258.1		HACA 01030908.1		HACA 1028197.1		HACA 01016812.1		HACA 01024270.1		HACA 01010127.1		HACA 01001173.1		HACA 01028341.1		HACA 1008519.1	
IoA-00	**F**	**PEG300**	**0**	24.630	15.675	23.385	16.044	20.160	48.166	20.895	45.411	21.070	38.395	20.445	50.415	28.620	9.904	19.360	50.576	26.290	42.545	22.485	39.035
IoA-00	**F**	**PEG300**	**0**	25.140	11.450	23.380	16.148	20.380	41.608	22.380	16.449	22.285	17.498	20.575	46.306	28.475	10.889	19.745	39.104	26.155	46.800	23.190	24.608
IoA-00	**F**	**PEG300**	**0**	25.435	9.592	23.090	19.445	19.905	56.548	22.835	12.060	22.620	14.103	20.440	50.558	28.450	11.270	19.330	51.501	25.925	55.207	22.585	36.603
IoA-00	**F**	**PEG300**	**0**	26.250	5.792	23.515	14.708	18.875	108.54	22.035	20.852	22.025	20.702	20.730	41.795	28.015	14.880	19.850	36.523	26.630	33.763	23.290	23.089
IoA-00	**F**	**PEG300**	**0**	25.460	9.409	23.610	13.804	19.520	72.085	21.550	28.959	21.910	22.420	20.540	47.364	29.030	7.495	19.655	41.511	25.915	55.448	22.420	40.723
IoA-00	**F**	**PEG300**	**0**	25.120	11.592	23.055	19.911	19.090	94.481	21.250	35.506	21.385	31.322	20.095	63.463	27.835	16.828	19.560	44.430	26.025	51.327	22.255	45.365
IoA-00	**M**	**PEG300**	**0**	24.575	16.215	21.470	56.450	22.085	14.303	21.050	40.688	21.075	38.396	20.605	45.363	28.565	10.247	20.430	24.840	26.490	36.972	22.805	31.624
IoA-00	**M**	**PEG300**	**0**	25.040	12.201	21.440	57.589	22.305	12.454	20.130	76.164	20.125	70.794	20.490	48.955	26.135	52.333	19.670	41.103	26.560	35.161	22.685	34.213
IoA-00	**M**	**PEG300**	**0**	24.795	14.184	22.040	38.777	22.875	8.736	21.050	40.688	21.200	35.299	21.115	32.465	28.135	13.681	20.030	32.372	27.310	20.786	22.875	30.289
IoA-00	**M**	**PEG300**	**0**	23.915	24.393	21.415	58.506	22.210	13.202	20.345	65.819	20.435	57.898	20.770	40.815	28.050	14.530	20.160	29.714	26.855	28.656	22.955	28.667
IoA-00	**M**	**PEG300**	**0**	21.920	83.161	21.840	44.392	24.580	2.960	20.220	71.785	20.560	53.416	21.130	32.163	28.200	13.147	20.095	31.013	26.260	43.482	23.475	20.394
IoA-00	**M**	**PEG300**	**0**	24.410	17.982	21.295	63.331	20.625	36.176	21.940	22.192	21.740	24.911	20.190	59.741	27.385	23.021	20.115	30.604	27.010	25.748	22.890	30.311
IoA-00	**F**	**DTM**	**0.05**	25.320	10.252	22.995	20.737	19.740	62.760	21.520	29.639	21.650	29.381	20.220	58.397	28.250	12.677	19.410	48.877	25.465	76.842	22.590	36.413
IoA-00	**F**	**DTM**	**0.05**	24.825	13.920	22.850	22.869	17.935	196.07	21.265	35.218	21.485	29.360	21.235	30.033	28.605	9.976	19.730	39.517	27.385	19.631	22.750	32.785
IoA-00	**F**	**DTM**	**0.05**	25.240	10.801	23.205	18.021	18.380	147.97	21.805	24.339	21.935	21.962	20.890	37.744	29.130	7.130	19.840	36.718	26.595	34.335	23.300	23.052
IoA-00	**F**	**DTM**	**0.05**	26.555	4.813	23.080	19.567	18.915	105.7	22.210	18.462	22.050	20.372	20.515	48.121	28.670	9.541	20.025	32.482	26.475	37.351	22.995	27.924
IoA-00	**F**	**DTM**	**0.05**	24.450	17.687	23.550	14.385	19.300	83.036	21.320	33.880	21.470	29.652	20.200	59.213	27.940	15.634	19.465	47.163	27.080	24.399	21.735	64.439
IoA-00	**F**	**DTM**	**0.05**	24.160	20.936	23.140	18.812	19.940	55.267	21.070	40.137	21.200	35.305	20.335	54.153	28.500	10.749	19.110	59.629	25.860	57.965	21.860	58.780
IoA-00	**M**	**DTM**	**0.05**	22.845	47.077	21.795	45.974	22.805	9.101	19.590	110.007	20.155	69.494	20.550	47.054	27.965	15.340	19.255	54.179	26.975	26.425	21.845	59.400
IoA-00	**M**	**DTM**	**0.05**	23.575	30.073	20.300	121.89	23.145	7.324	19.515	115.774	20.340	61.782	20.715	42.206	27.010	29.794	19.285	53.076	25.140	95.910	22.840	30.911
IoA-00	**M**	**DTM**	**0.05**	24.070	22.127	20.230	127.56	22.030	14.786	20.505	59.162	20.745	47.383	20.165	60.542	27.620	19.433	19.930	34.599	25.645	67.189	22.855	30.606
IoA-00	**M**	**DTM**	**0.05**	22.215	69.355	20.895	83.264	23.340	6.472	20.540	57.596	20.955	41.541	21.065	33.577	27.745	17.765	18.995	64.478	25.660	66.389	22.745	33.085
IoA-00	**M**	**DTM**	**0.05**	23.135	39.352	21.385	59.665	23.535	5.722	19.980	84.499	20.270	64.578	21.065	33.720	27.965	15.410	19.280	53.249	26.135	47.596	21.875	58.468
IoA-00	**M**	**DTM**	**0.05**	23.740	27.163	21.235	65.861	23.445	6.058	19.565	111.949	20.290	63.609	20.690	43.035	27.980	15.163	19.150	58.081	25.920	55.417	22.015	53.248
IoA-00	**F**	**EMB**	**25**	23.405	33.405	23.300	16.971	20.200	46.982	21.080	39.910	21.235	34.517	20.145	61.349	27.590	19.775	19.135	58.631	26.830	29.288	21.545	72.478
IoA-00	**F**	**EMB**	**25**	24.100	21.755	23.130	18.936	20.060	51.247	20.540	57.699	20.595	52.282	20.480	49.307	28.360	11.751	18.950	66.271	25.940	54.741	22.285	44.628

Supplementary material

Strain	Sex	Drug	Dose	Av. Ct value HACA 01002875.1	Relat. copy no.	Av. Ct value HACA 01023258.1	Relat. copy no.	Av. Ct value HACA 01030908.1	Relat. copy no.	Av. Ct value HACA 1028197.1	Relat. copy no.	Av. Ct value HACA 01016812.1	Relat. copy no.	Av. Ct value HACA 01024270.1	Relat. copy no.	Av. Ct value HACA 01010127.1	Relat. copy no.	Av. Ct value HACA 01001173.1	Relat. copy no.	Av. Ct value HACA 01028341.1	Relat. copy no.	Av. Ct value HACA 1008519.1	Relat. copy no.
IoA-00	**F**	**EMB**	**25**	23.365	34.166	22.625	26.452	19.450	75.623	20.620	54.536	21.145	36.586	19.910	71.719	28.275	12.475	18.740	76.207	25.770	61.449	21.540	72.566
IoA-00	**F**	**EMB**	**25**	21.870	85.734	22.540	28.200	19.200	91.547	21.455	31.195	21.405	30.935	19.945	69.971	28.335	12.068	19.195	56.335	25.950	54.255	22.135	49.110
IoA-00	**F**	**EMB**	**25**	27.550	2.597	27.335	1.191	24.565	2.990	25.865	1.539	25.270	2.538	25.925	1.388	NA	NA	23.675	2.883	32.400	0.568	26.565	2.716
IoA-00	**F**	**EMB**	**25**	24.435	17.744	22.830	23.092	18.970	102.11	19.930	87.271	20.215	66.751	20.025	66.374	28.165	13.397	19.005	64.153	25.215	91.069	21.495	75.069
IoA-00	**M**	**EMB**	**25**	24.800	14.392	20.280	124.03	20.940	29.431	19.800	95.565	20.120	71.031	20.135	61.753	27.170	26.267	19.470	46.936	25.235	89.725	22.095	50.396
IoA-00	**M**	**EMB**	**25**	24.160	20.934	20.850	84.900	21.475	20.986	19.380	127.221	20.075	73.084	20.770	40.723	27.090	27.632	18.990	64.587	25.530	72.774	22.135	49.110
IoA-00	**M**	**EMB**	**25**	25.415	9.668	20.780	89.325	21.925	15.801	20.300	67.820	20.730	47.842	20.360	53.300	27.960	15.384	19.800	37.803	26.020	51.564	22.310	43.756
IoA-00	**M**	**EMB**	**25**	24.105	21.663	20.670	95.518	20.800	32.593	20.025	83.566	20.190	67.927	19.710	81.642	26.855	33.526	19.020	63.286	25.340	83.257	21.675	66.908
IoA-00	**M**	**EMB**	**25**	23.205	37.690	21.805	45.495	22.540	10.722	20.400	63.501	20.485	56.076	21.090	33.140	27.995	15.014	19.940	34.551	26.435	38.529	22.615	36.030
IoA-00	**M**	**EMB**	**25**	23.150	38.989	21.065	73.894	22.630	10.172	20.580	56.090	20.880	43.525	20.900	37.562	27.580	19.897	19.715	39.987	25.645	67.189	22.735	33.123
IoA-02	**F**	**PEG300**	**0**	22.800	51.530	23.160	22.639	19.835	50.512	21.825	22.263	21.640	27.076	20.820	46.625	27.515	19.914	19.435	50.415	26.210	41.479	21.910	62.936
IoA-02	**F**	**PEG300**	**0**	23.285	38.293	23.830	14.640	20.505	33.978	22.005	19.748	22.515	15.541	21.285	33.751	27.300	23.062	20.010	34.554	27.870	13.854	22.765	37.212
IoA-02	**F**	**PEG300**	**0**	23.820	27.885	23.280	20.893	19.445	65.141	20.755	44.235	20.490	56.150	20.390	60.439	26.820	32.035	19.055	64.649	26.195	41.781	21.665	72.656
IoA-02	**F**	**PEG300**	**0**	22.075	78.590	22.445	36.763	19.150	78.473	21.175	33.738	21.180	36.243	20.310	63.418	26.920	29.797	19.290	55.487	24.980	97.258	22.610	41.037
IoA-02	**F**	**PEG300**	**0**	22.210	72.567	22.730	30.382	17.665	203.15	20.915	40.087	20.985	41.032	20.940	42.158	26.825	32.526	19.550	46.730	25.540	65.878	23.165	29.126
IoA-02	**F**	**PEG300**	**0**	24.035	24.487	23.550	17.403	19.255	73.402	21.180	33.638	21.540	28.846	20.220	67.129	26.395	43.122	19.150	60.824	26.300	38.940	21.695	71.354
IoA-02	**M**	**PEG300**	**0**	23.430	35.115	21.085	91.919	20.315	37.207	21.535	26.772	21.620	27.472	20.310	63.343	25.080	108.01	19.670	44.023	26.640	31.274	21.890	63.557
IoA-02	**M**	**PEG300**	**0**	25.140	12.979	22.025	48.718	22.755	7.762	21.850	21.824	22.265	18.237	22.040	20.728	26.995	28.593	21.505	12.964	26.875	26.083	23.755	20.360
IoA-02	**M**	**PEG300**	**0**	22.050	80.041	21.275	81.043	22.270	10.594	20.185	64.020	19.925	80.523	20.425	58.744	25.175	101.33	19.780	40.200	26.035	46.767	22.685	39.023
IoA-02	**M**	**PEG300**	**0**	23.425	35.243	20.595	128.04	22.730	7.889	20.340	57.856	21.085	38.636	20.570	53.547	25.560	78.277	20.395	26.872	26.160	42.807	23.640	21.888
IoA-02	**M**	**PEG300**	**0**	22.820	50.492	21.210	84.536	22.725	7.913	20.465	53.334	20.455	57.429	21.050	39.279	25.910	60.416	20.125	32.277	26.315	38.451	22.760	37.376
IoA-02	**M**	**PEG300**	**0**	22.960	46.498	21.185	85.917	22.310	10.352	21.395	34.633	20.510	55.437	20.565	53.679	24.880	124.67	19.970	35.507	25.470	70.298	22.800	36.379
IoA-02	**F**	**DTM**	**0.05**	21.820	91.488	22.660	31.802	19.215	75.217	20.670	46.725	20.800	46.304	19.725	92.255	26.855	31.505	18.795	76.817	25.475	69.074	22.005	59.076
IoA-02	**F**	**DTM**	**0.05**	22.975	46.013	22.530	34.642	17.095	293.47	19.695	88.173	20.005	76.456	20.965	41.464	26.530	39.172	19.230	57.785	25.755	56.734	21.755	68.770
IoA-02	**F**	**DTM**	**0.05**	21.845	90.131	23.010	25.051	19.670	56.201	20.820	42.430	21.105	38.078	20.415	59.341	26.120	52.205	19.240	57.289	26.070	45.602	22.360	47.562
IoA-02	**F**	**DTM**	**0.05**	22.135	75.928	22.910	26.807	19.020	85.187	20.855	41.697	20.880	43.862	19.975	78.696	26.445	41.621	18.980	68.896	26.815	27.135	21.215	95.694
IoA-02	**F**	**DTM**	**0.05**	21.465	113.0	22.945	26.187	19.275	72.645	21.370	29.749	21.270	34.233	20.440	58.190	25.180	100.71	19.190	59.493	26.670	30.103	21.395	85.623
IoA-02	**F**	**DTM**	**0.05**	21.570	106.16	23.075	23.977	19.175	77.267	20.865	41.195	20.760	47.308	20.075	73.615	26.690	35.014	18.625	85.717	25.560	65.369	21.570	76.958

Supplementary material

Strain	Sex	Drug	Dose	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.
				HACA 01002875.1		HACA 01023258.1		HACA 01030908.1		HACA 1028197.1		HACA 01016812.1		HACA 01024270.1		HACA 01010127.1		HACA 01001173.1		HACA 01028341.1		HACA 1008519.1	
IoA-02	M	DTM	0.05	22.075	78.645	20.715	118.05	20.535	32.239	20.065	69.095	20.145	69.880	21.040	39.556	23.675	290.65	19.220	58.165	25.825	54.385	22.385	47.025
IoA-02	M	DTM	0.05	22.150	75.169	21.030	95.385	21.545	16.891	19.995	72.204	19.870	83.682	20.380	60.467	24.535	158.27	19.410	51.270	25.935	50.663	21.885	63.586
IoA-02	M	DTM	0.05	21.650	101.21	20.860	107.03	21.470	17.734	19.400	105.985	19.135	132.62	20.200	67.916	24.890	123.36	18.540	90.787	24.960	98.678	21.730	69.810
IoA-02	M	DTM	0.05	23.325	37.363	20.215	165.38	22.395	9.789	20.270	60.547	20.600	52.361	19.605	100.24	24.430	171.72	19.435	50.526	24.560	130.412	22.350	47.979
IoA-02	M	DTM	0.05	23.190	40.486	20.695	119.90	19.540	61.037	20.770	43.909	20.550	54.087	20.190	68.351	24.065	219.99	19.655	43.612	25.630	62.094	21.275	92.140
IoA-02	M	DTM	0.05	24.375	20.011	21.485	70.761	20.700	29.060	19.985	72.678	20.070	73.308	20.135	70.880	23.905	247.06	19.280	55.794	26.700	29.578	21.625	74.498
IoA-02	F	EMB	25	23.090	42.988	23.405	19.226	19.755	53.172	20.975	38.431	20.815	45.697	20.835	45.173	27.060	27.095	19.135	61.349	26.175	42.366	21.850	65.527
IoA-02	F	EMB	25	21.765	95.078	22.310	40.197	19.430	65.521	20.360	57.114	20.560	53.776	20.550	54.259	27.060	27.026	19.010	66.588	25.770	56.856	21.860	64.571
IoA-02	F	EMB	25	21.130	137.93	22.385	38.319	19.055	83.305	20.960	38.798	20.835	45.137	20.265	65.198	26.615	37.967	18.650	84.364	25.005	95.584	21.855	64.692
IoA-02	F	EMB	25	23.370	36.384	23.315	20.391	19.200	75.936	21.070	36.105	21.420	31.128	20.190	68.491	26.190	49.676	19.140	61.161	26.185	42.066	21.140	100.030
IoA-02	F	EMB	25	22.250	70.817	23.050	24.397	18.905	91.729	20.100	67.508	20.595	52.530	19.995	77.600	26.510	39.778	18.965	68.661	25.990	49.087	21.295	91.062
IoA-02	F	EMB	25	22.810	50.752	22.175	44.208	18.520	117.68	21.240	32.517	21.135	37.308	20.305	63.463	26.465	40.970	18.735	81.244	24.820	108.730	22.280	50.011
IoA-02	M	EMB	25	22.970	46.181	20.955	100.44	21.295	19.823	19.890	77.455	20.505	55.817	20.440	58.171	24.445	169.85	19.745	41.382	26.000	47.836	22.330	48.441
IoA-02	M	EMB	25	22.335	67.324	20.740	116.40	20.440	34.333	20.620	48.272	20.450	57.589	20.145	70.362	24.075	218.24	19.455	49.771	25.690	59.672	21.810	66.494
IoA-02	M	EMB	25	22.115	76.796	20.730	116.80	20.800	27.198	19.515	98.413	19.325	117.6	20.205	67.700	24.635	147.73	18.705	81.366	25.410	72.286	22.040	57.807
IoA-02	M	EMB	25	22.895	48.308	21.170	87.167	21.485	17.529	20.655	47.171	20.415	58.895	26.700	1.026	24.550	156.89	19.675	43.082	25.715	58.369	22.045	57.624
IoA-02	M	EMB	25	22.345	67.010	21.235	83.057	21.190	21.291	19.475	101.250	19.510	104.71	20.310	63.260	24.895	122.93	18.470	95.181	25.615	62.537	21.560	77.426
IoA-02	M	EMB	25	22.400	64.788	20.740	116.11	20.035	44.426	20.315	58.993	20.090	72.367	20.050	74.808	25.210	98.697	19.300	55.091	26.135	43.836	21.170	98.410
IoA-02	F	DTM	2	21.795	93.799	22.495	25.635	19.515	59.519	21.605	22.797	21.295	29.736	20.605	47.885	26.995	22.762	19.085	59.714	25.955	50.104	21.855	52.465
IoA-02	F	DTM	2	22.690	53.965	22.650	23.158	17.465	210.28	20.850	37.572	20.525	49.057	21.140	34.219	27.120	20.671	19.540	44.497	26.000	48.585	21.820	53.706
IoA-02	F	DTM	2	22.025	81.458	22.865	20.111	19.290	68.274	20.645	43.083	20.825	40.420	20.310	57.577	26.185	39.508	19.055	60.910	26.045	47.190	21.975	48.646
IoA-02	F	DTM	2	23.515	32.414	22.455	26.379	17.655	186.81	20.595	44.462	20.510	49.573	20.325	57.036	26.270	37.291	19.355	50.121	25.105	86.546	21.875	51.905
IoA-02	F	DTM	2	22.285	69.260	22.885	19.872	18.705	97.869	21.160	30.627	20.545	48.533	20.250	59.877	27.640	14.504	19.035	61.952	25.070	88.466	21.355	71.625
IoA-02	F	DTM	2	22.170	74.675	22.625	23.541	19.780	50.494	20.810	38.620	21.210	31.418	20.650	46.546	26.580	30.071	19.170	56.567	25.725	58.651	21.935	49.889
IoA-02	M	DTM	2	21.885	88.679	21.090	64.523	23.020	6.869	19.070	122.024	19.220	114.64	20.615	47.598	25.205	77.706	18.760	74.695	26.015	48.092	21.960	49.113
IoA-02	M	DTM	2	23.285	37.358	21.185	60.568	20.935	24.858	22.015	17.378	22.185	16.688	21.550	26.497	24.775	104.61	19.410	48.412	25.680	59.815	21.300	74.119
IoA-02	M	DTM	2	23.425	34.403	21.350	54.388	21.805	14.579	21.025	33.454	20.550	48.263	20.155	63.453	25.620	58.482	19.820	37.269	26.100	45.769	22.280	40.211
IoA-02	M	DTM	2	22.130	76.390	20.440	98.716	21.035	23.325	20.390	50.949	19.590	90.133	20.240	60.168	25.310	72.316	18.750	74.359	25.225	80.450	21.595	61.658

Supplementary material

Strain	Sex	Drug	Dose	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.	Av. Ct value	Relat. copy no.
				HACA 01002875.1		HACA 01023258.1		HACA 01030908.1		HACA 1028197.1		HACA 01016812.1		HACA 01024270.1		HACA 01010127.1		HACA 01001173.1		HACA 01028341.1		HACA 1008519.1	
IoA-02	**M**	**DTM**	**2**	22.435	63.159	21.055	66.087	21.455	18.010	20.535	46.265	20.450	51.639	20.850	41.031	24.665	112.87	19.170	56.513	25.570	64.085	21.930	50.190
IoA-02	**M**	**DTM**	**2**	22.930	46.555	21.440	51.349	21.955	13.251	20.735	40.592	20.885	38.824	20.795	42.544	25.185	79.120	20.090	31.114	27.160	22.987	21.565	62.931
IoA-02	**F**	**EMB**	**150**	21.495	112.80	23.335	14.824	19.210	71.750	20.145	59.898	20.790	41.358	20.055	67.618	26.110	41.723	19.145	57.440	26.665	32.364	20.900	95.147
IoA-02	**F**	**EMB**	**150**	22.620	56.428	22.975	18.733	19.650	54.707	21.855	19.316	21.165	32.351	20.960	38.383	26.795	26.533	19.130	58.024	25.725	58.353	22.715	30.685
IoA-02	**F**	**EMB**	**150**	21.155	139.17	22.305	29.038	19.265	69.331	21.390	26.358	20.450	51.507	20.830	41.547	27.070	21.409	19.085	59.712	25.695	59.309	21.540	64.211
IoA-02	**F**	**EMB**	**150**	21.530	110.43	23.085	17.424	19.300	67.851	20.320	53.354	20.635	45.722	20.330	56.990	26.720	27.296	19.060	60.689	25.985	49.038	21.485	66.044
IoA-02	**F**	**EMB**	**150**	21.825	92.040	22.865	20.243	19.205	71.978	21.185	30.105	20.925	37.985	20.285	58.518	26.595	30.666	19.155	57.128	26.425	36.940	21.175	80.177
IoA-02	**F**	**EMB**	**150**	22.015	82.612	22.450	26.439	19.410	63.412	20.240	56.313	20.095	64.927	20.400	54.422	25.915	47.703	18.975	64.184	25.350	74.151	21.505	65.243
IoA-02	**M**	**EMB**	**150**	21.730	97.568	21.185	60.635	21.195	21.128	19.510	91.185	19.865	75.532	21.025	36.857	24.130	163.53	19.230	54.356	25.965	49.699	22.140	43.902
IoA-02	**M**	**EMB**	**150**	21.940	85.779	20.755	80.271	21.355	19.149	19.945	68.400	19.620	88.391	20.305	57.788	24.645	114.93	19.025	62.097	25.395	71.845	21.955	49.280
IoA-02	**M**	**EMB**	**150**	22.665	54.810	20.300	108.20	21.740	15.113	19.580	87.035	19.480	96.851	19.995	70.253	24.375	137.99	18.985	63.753	25.245	79.034	22.245	41.114
IoA-02	**M**	**EMB**	**150**	23.110	41.633	20.775	79.450	20.790	27.163	21.205	29.798	20.345	55.155	20.550	49.587	25.530	62.177	19.390	49.142	25.495	67.259	21.555	63.214
IoA-02	**M**	**EMB**	**150**	22.135	76.009	20.790	78.780	21.675	15.785	20.110	61.291	20.635	45.679	21.045	36.303	25.070	85.347	19.285	52.454	25.795	55.909	21.295	74.358
IoA-02	**M**	**EMB**	**150**	21.335	124.55	20.350	105.08	21.490	17.628	19.780	76.300	19.300	108.86	20.280	58.668	24.885	97.006	18.705	76.421	25.655	60.965	22.195	42.447

F: Female, M: Male, PEG300: Polyethylene glycol, M_n = 300, DTM: Deltamethrin, EMB: Emamectin benzoate, Av. Ct value: Average cycle threshold value, Relat. copy no.: Average relative copy number.

Table S4.7 Rating of *L. salmonis* in bioassays with Polyethylene glycol, M_n = 300 (PEG300), emamectin benzoate (EMB), and deltamethrin (DTM).

Compound	Strain	Concentration	Female			Male		
			Live	Weak	Moribund	Live	Weak	Moribund
PEG300	IoA-00	0.05%	8	1	0	9	0	0
PEG300	IoA-02	0.05%	8	0	1	8	0	0
EMB	IoA-00	25 µg L^{-1}	8	0	0	8	0	0
EMB	IoA-02	25 µg L^{-1}	7	1	0	8	0	0
EMB	IoA-00	150 µg L^{-1}	0	0	8	0	0	8
EMB	IoA-02	150 µg L^{-1}	8	0	0	8	0	0
DTM	IoA-00	0.05 µg L^{-1}	8	0	1	8	0	0
DTM	IoA-02	0.05 µg L^{-1}	7	0	1	8	0	0
DTM	IoA-00	2 µg L^{-1}	0	0	9	0	0	7
DTM	IoA-02	2 µg L^{-1}	8	0	0	8	0	0

Table S4.8 Single nucleotide polymorphism (SNP) loci within carboxylesterase (CaE) genes that showed significantly different genotype frequencies in two *L. salmonis* strains. SNP analysis in CaE genes were performed based on RNA-seq data of 15 adult male salmon lice of the drug susceptible strain IoA-00 and the multi-resistant strain IoA-02. *P*-values represent pairwise comparisons of genotype frequencies between both strains using the Fisher's exact probability test. Genotype frequencies were regarded significantly different between strains when the $P \leq 0.05$.

Clade	NCBI accession no.	SNP locus	Allele 1	Allele 2	Strain	n	Genotypes (%)			Frequency allele 2	Genotypic differentiation Fisher's exact probability test	Corresponding amino acid
							gg	ga	aa			
O	**HACA01008519.1**	**358**	**A**	**G**	IoA-00	8	12.50	37.50	50.0	0.67	1.24E-02	P95P
					IoA-02	7	71.43	28.57	0.0	0.14		
		1193	**C**	**G**	IoA-00	8	100.0	0.0	0.0	0.0	2.00E-04	L374V
					IoA-02	7	0.0	0.0	100.0	1.0		
		1197	**T**	**A**	IoA-00	8	100.0	0.0	0.0	0.0	2.00E-04	L375Q
					IoA-02	7	0.0	0.0	100.0	1.0		
		1814	**G**	**T**	IoA-00	8	100.0	0.0	0.0	0.0	2.00E-04	E581*
					IoA-02	7	0.0	100.0	0.0	0.5		
O	**HACA01024270.1**	**929**	**T**	**G**	IoA-00	8	100.0	0.0	0.0	0.0	1.30E-04	S148R
					IoA-02	7	0.00	85.71	14.29	0.57		
		931	**T**	**C**	IoA-00	8	100.0	0.0	0.0	0.0	2.00E-04	F149S
					IoA-02	7	0.00	100.0	0.0	0.5		
		933	**C**	**T**	IoA-00	8	100.0	0.0	0.0	0.0	2.00E-04	Q150*
					IoA-02	7	0.0	100.0	0.0	0.5		
		934	**A**	**G**	IoA-00	8	100.0	0.0	0.0	0.0	2.00E-04	Q150R
					IoA-02	7	0.0	100.0	0.0	0.5		
		939	**T**	**C**	IoA-00	8	100.0	0.0	0.0	0.0	2.00E-04	S152P
					IoA-02	7	0.0	100.0	0.0	0.5		
1A	**HACA01023258.1**	**1086**	**T**	**A**	IoA-00	8	100.0	0.0	0.0	0	2.00E-04	F362Y
					IoA-02	7	0.0	0.00	100.0	1		
		1292	**C**	**A**	IoA-00	6	33.33	50.00	16.67	0.417	2.25E-02	K431Q
					IoA-02	7	100.0	0.0	0.0	0		

Clade	NCBI accession no.	SNP locus	Allele 1	Allele 2	Strain	n	Genotypes (%)			Frequency allele 2	Genotypic differentiation Fisher's exact probability test	Corresponding amino acid
							gg	ga	aa			
1B	**HACA01002875.1**	**308**	**G**	**T**	IoA-00	8	12.50	0.0	87.5	0.875	1.46E-03	I88M
					IoA-02	7	100.0	0.0	0.0	0		
		1746	**T**	**C**	IoA-00	8	0.0	37.5	62.5	0.813	2.30E-04	S568P
					IoA-02	7	100.0	0.0	0.0	0		
		1780	**T**	**A**	IoA-00	8	100.0	0.0	0.0	0	2.00E-04	V579E
					IoA-02	7	0.0	100.0	0.0	0.5		
H	**HACA01028197.1**	**1015**	**T**	**A**	IoA-00	8	75.0	25.0	0.0	0.125	4.10E-02	A338A
					IoA-02	7	14.29	85.71	0.0	0.429		

Table S4.9 Number of carboxylesterase (CaE) family members[†] in eight arthropods.

	Phylum	**Arthropods**							
	Clade	Mandibulata - Altocrustacea							
	Subphylum	**Hexapoda**						**Crustacea**	**Chelicerata**
	(Sub)class	Insecta						Copepoda	Arachnida
	Species	***A. mellifera***	***D. melanogaster***	***A. gambiae***	***B. mori***	***T. castaneum***	***P. humanus***	***L. salmonis***	***T. urticae***
Dietary/detoxification class									
Clade A, B & C		8	13	16	55	26	3	-	-
Hormone/semiochemical processing class									
Clade D (Integument esterases)		1	3	-	2	2	-	-	-
Clade E (Secreted β esterases)		2	2	5	2	7	1	1	-
Clade F & G (JHE)		2	3	9	4	2	-	-	2
Clade H (Glutactins)		1	5	10	1	1	1	2	2
Neuro/developmental class									
Clade I (Uncharacterized clade)		1	1	1	1	1	1	2	-
Clade J (Acetylcholinesterases)		2	1	2	2	2	2	2	1
Clade K (Gliotactins)		1	1	1	1	1	1	1	1
Clade L (Neuroligins)		5	4	5	6	5	5	6	5
Clade M (Neurotactins)		1	2	2	2	2	3	2	1
Non-insect clades									
[‡]Clade O		-	-	-	-	-	-	5	-
[§]Clade J'		-	-	-	-	-	-	-	34
[§]Clade J''		-	-	-	-	-	-	-	22
[§]Undetermined		-	-			-	-	-	3
Total		**24**	**35**	**51**	**76**	**49**	**17**	**21**	**71**

[†]Numbers from Claudianos et al. (2006), Oakeshott et al. (2005), Grbić et al. (2011), Yu et al. (2009), Lee et al. (2010), Oakeshott et al. (2010), and this study.

[‡]Labelling according to this study.

[§]Labelling according to Grbić et al. (2011).

Table S4.10 Summary of carboxylesterase (CaE) sequences within the new clade O in *L. salmonis*. CaEs were identified by homology searches in transcriptome (EBI ENA reference ERS237607) and genome assemblies (LSalAtl2s, ensemble.metazoa.org), using the entire complement of *Drosophila melanogaster* as queries. The assignment of sequences to clades is based on the phylogenetic analysis of *L. salmonis* CaEs shown in Fig. 1. The coding sequence of the *L. salmonis* CaEs within the new clade O were annotated using BLASTp searches against the NCBI Non-Redundant Protein Sequence Collection of *Copepoda*, and aligned with *Tetranychus urticae* (class Arachnida) CaE sequences (Grbić et al., 2011).

Lepeophtheirus salmonis	**Annotation in *Copepoda***					**Annotation in *Tetranychus urticae*†**					
NCBI accession no.	**BLAST hit**	**NCBI accession no.**	**Species**	**E-value**	**Identity (%)**	**Best BLAST hit**	***T. urticae* gene ID**	**NCBI accession no.**	**E-value**	**Identity (%)**	***T. urticae* clade**
HACA01024270.1	hypothetical protein TCAL_10027	TRY70685.1	*Tigriopus californicus*	7.00 E-119	41.07%	Carboxylesterase 4A isoform X1	tetur04g06380	XP_015782259.1	1.00E-48	29.68%	F'
HACA01001173.1	Fatty acyl-CoA hydrolase precursor	XP_023334367.1	*Eurytemora affinis*	1E-85	32.62%	Esterase E4 isoform X1	tetur23g00910	XP_015790984.1	5.00E-61	33.21%	F'
HACA01008519.1	hypothetical protein TCAL_05343	TRY76473.1	*Tigriopus californicus*	9.00 E-123	36.47%	Carboxylesterase 4A isoform X1	tetur04g06380	XP_015782259.1	3.00E-74	33.54%	F'
HACA01010127.1	hypothetical protein TCAL_02277	TRY67019.1	*Tigriopus californicus*	7.00 E-25	31.62%	Carboxylesterase 4A isoform X1	tetur04g06380	XP_015782259.1	0.001	22.22%	F'
HACA01028341.1	hypothetical protein TCAL_11235	TRY61787.1	*Tigriopus californicus*	1.00 E-40	28.83%	Esterase E4 isoform X1	tetur23g00910	XP_015790984.1	2.00E-20	26.79%	F'

†The *T. urticae* CaE gene family contains 71 genes with two new clades representing 34 (clade J’) and 22 (clade J’’) CaEs, respectively (Grbić et al., 2011).

Research Article

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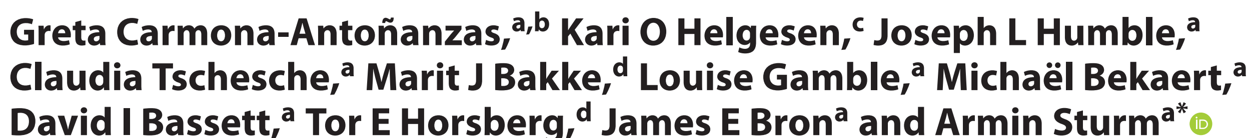
Mutations in voltage-gated sodium channels from pyrethroid resistant salmon lice (*Lepeophtheirus salmonis*)



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Abstract

BACKGROUND: Parasitic salmon lice (*Lepeophtheirus salmonis*) cause high economic losses in Atlantic salmon farming. Pyrethroids, which block arthropod voltage-gated sodium channels (Na_v1), are used for salmon delousing. However, pyrethroid resistance is common in *L. salmonis*. The present study characterized *Na_v1* homologues in *L. salmonis* in order to identify channel mutations associated to resistance, called kdr (knockdown) mutations.

RESULTS: Genome scans identified three *L. salmonis Na_v1* homologues, *$LsNa_v1.1$*, *$LsNa_v1.2$* and *$LsNa_v1.3$*. Arthropod *kdr* mutations map to specific Na_v1 regions within domains DI-III, namely segments S5 and S6 and the linker helix connecting S4 and S5. The above channel regions were amplified by RT-PCR and sequenced in deltamethrin-susceptible and deltamethrin-resistant *L. salmonis*. While *$LsNa_v1.1$* and *$LsNa_v1.2$* lacked nucleotide polymorphisms showing association to resistance, *$LsNa_v1.3$* showed a non-synonymous mutation in S5 of DII occurring in deltamethrin-resistant parasites. The mutation is homologous to a previously described kdr mutation (I936V, numbering according to *Musca domestica* Vssc1) and was present in two pyrethroid-resistant *L. salmonis* strains (allele frequencies of 0.800 and 0.357), but absent in two pyrethroid-susceptible strains.

CONCLUSIONS: The present study indicates that a kdr-mutation in *$LsNa_V$ 1.3* may contribute to deltamethrin resistance in *L. salmonis*.
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Supporting information may be found in the online version of this article.

Keywords: deltamethrin; sea lice; sodium channel; resistance

1 INTRODUCTION

Voltage-gated sodium channels play essential roles in the initiation and propagation of action potentials in neurons, myocytes and other electrically excitable cells,[1] and are the pharmacological targets of different types of natural neurotoxins and synthetic chemicals interfering with channel gating to disrupt neurotransmission.[2] Such compounds include toxins from pufferfish, scorpions and cnidarians, local anaesthetics, and insecticides.[2–4] The widespread use of insecticides interacting with sodium channels, namely DDT and the pyrethroids, has led to the evolution of resistance in arthropod populations under selection pressure. Point mutations in voltage-gated sodium channels are a common mechanism of pyrethroid resistance.[5]

Pyrethroids are synthetic analogues to botanical insecticides of the class of pyrethrins, which are produced by flowers of the genus *Chrysanthemum*. A number of distinct structural traits of pyrethroids cause their greater stability and efficacy as pest control agents compared to pyrethrins.[6] Pyrethroids are widely used to control phytophagous insects, human-disease vectors and animal parasites. Pyrethroids accounted for 17% of global insecticides sales in 2013, which makes them the second most important insecticide class after neonicotinoids.[6,7] However, their efficacy to control arthropod pests is challenged by the evolution of pyrethroid resistance,[8,9] which constitutes a major threat to food security and human health, and can lead to extensive economic losses.[10]

The toxicity of pyrethroids is based on their blocking of neuronal voltage-gated sodium channels, which involves interaction

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with the pore-forming α-subunit of the channel called Na_v.[4,11] Na_v consists of four internally homologous domains DI-IV, which are arranged symmetrically to form a central pore.[2] Knockdown resistance (kdr) is a key mechanism of pyrethroid resistance in arthropods and results from non-synonymous point mutations of Na_v reducing the channel's sensitivity to pyrethroids and usually conferring cross-resistance to DDT.[2,4,12] Alternatively, pyrethroid resistance can result from genetic changes that lead to the enhanced expression of biochemical detoxification pathways, often involving cytochromes P450 (CYPs) and/or glutathione-S-transferases (GSTs).[9,13,14]

Since the mid-1990s, the pyrethroids cypermethrin and deltamethrin have been licensed as veterinary medicines to treat farmed Atlantic salmon (*Salmo salar* Linnaeus, 1758) and rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) suffering from infections by sea lice (Copepoda: Caligidae).[15,16] Sea lice are ectoparasites feeding on the mucus, skin and blood of host fish. At high infection densities, sea louse infections can give rise to skin lesions, osmoregulatory imbalances, growth and immune suppression, secondary infections and, if untreated, potentially death.[16] In the Northern hemisphere, most caligid infections of farmed salmon involve the salmon louse (*Lepeophtheirus salmonis*, (Krøyer, 1837)). Two allopatric subspecies of *L. salmonis* exist, of which *L. salmonis salmonis* (Krøyer, 1837) inhabits the North Atlantic while *L. salmonis oncorhynchi* Skern-Mauritzen, Torrissen and Glover, 2014 is found in the North-East Pacific.[17] Infection by the smaller species *Caligus elongatus* Nordmann, 1832 also occurs in the Northern hemisphere. In Chile, the sea louse species *Caligus rogercresseyi* Boxshall and Bravo, 2000 is a significant pathogen in commercial salmon production.[18]

Only a limited range of veterinary drugs are available as licensed salmon delousing treatments.[19] The repeated use of a restricted range of anti-parasitic drugs can favour the evolution of resistance in the parasite.[20] Resistance to pyrethroids has been reported for both *L. salmonis* and *C. rogercresseyi*[21,22] and resistance to pyrethroids in *L. salmonis* is currently common in the North Atlantic.[20] The mechanism of pyrethroid resistance in *L. salmonis* is at present unknown. In 2005, a kdr-type mutation in the *L. salmonis* Na_v1 homologue was proposed as a major determinant of pyrethroid resistance in this fish parasite[23]; however, no later studies providing supporting evidence for this mechanism exist. Similarly, the involvement of cytochrome P450 monooxygenases in the detoxification of pyrethroids in *L. salmonis* has been demonstrated,[24] but its relation to resistance is unknown. Recently, it has been demonstrated that pyrethroid resistance is inherited maternally in *L. salmonis*.[25,26] While the results suggested that both mitochondrial and nuclear genetic determinants contributed to the resistance, the molecular mechanism causing resistance remains to be identified.

The aim of the present study was to assess potential roles for Na_v in determining the resistance of *L. salmonis* against pyrethroids by comparing nucleotide sequences and expression levels of Na_v1 homologues between two well characterized laboratory-maintained strains of the parasite, the deltamethrin-resistant strain IoA-02 and the deltamethrin-susceptible strain IoA-00. The expression of candidate single nucleotide polymorphisms identified by this approach was further investigated using allele-specific genotyping assays, taking into account the above and two further parasite strains, one of which was deltamethrin resistant.

2 MATERIALS AND METHODS

2.1 Ethics statement

All research projects involving the Institute of Aquaculture (IoA) are subjected to a thorough Ethical Review Process prior to any work being approved. All projects with IoA participation are required to be submitted to the IoA Ethical Committee for approval, irrespective of where experimentation will be carried out. This procedure ensures all ethical issues are addressed before an experiment can be initiated. The present research was assessed by the IoA Ethical Review Committee and passed the Ethical Review Process of the University of Stirling (Project ID ASPA10/2013). Laboratory infections of Atlantic salmon with *L. salmonis* were carried out under UK Home Office project license 645 PPL 60/4522.

2.2 *Lepeophtheirus salmonis* strains and husbandry

The laboratory-maintained strains of *L. salmonis* used in this study were established from egg strings collected from Scottish salmon production sites.[26,27] No drug selection has been applied during the isolation or maintenance of the strains. Between their isolation and the year 2015, in which the experiments described in this report were carried out, strains have shown stable drug susceptibility profiles in bioassays (data not shown). Strain IoA-00 was established in 2003 from an isolate originating in the Firth of Clyde and is susceptible to all current delousing agents including deltamethrin. Strain IoA-01, derived in 2008 from material collected in Sutherland, is susceptible to deltamethrin[26] but resistant to emamectin benzoate.[27] Strains IoA-02 and IoA-03, which were established in 2011 and 2014, respectively, from the Shetland Islands and Sutherland, are resistant to emamectin benzoate and deltamethrin.[26,27] In bioassays involving 30 min of pesticide exposure and 24 h of recovery, the following deltamethrin median effective concentrations (EC_{50}s), followed by 95% confidence limits, were determined in a previous study[26]: IoA-00: 0.28 $\mu g\,L^{-1}$ (0.23–0.36 $\mu g\,L^{-1}$), IoA-01: 0.36 $\mu g\,L^{-1}$ (0.26–0.46 $\mu g\,L^{-1}$), IoA-02: 40.1 $\mu g\,L^{-1}$ (22.1–158.9 $\mu g\,L^{-1}$), IoA-03: >2.0 $\mu g\,L^{-1}$. Under culture, strains have been maintained under identical conditions as described in detail elsewhere.[28] In brief, parasites were maintained on Atlantic salmon hosts kept in circular tanks supplied with fresh seawater at ambient temperature, using a photoperiod corresponding to natural day length. To propagate cultures, egg strings were obtained from gravid females, hatched and allowed to develop to copepodids, which were used to infect naïve host fish. Infection rates were maintained at levels that were unlikely to compromise fish welfare. Prior to the collection of *L. salmonis* from hosts, host fish were euthanized under a UK Home Office approved Schedule 1 method. All laboratory infections were carried out under UK Home Office licence and appropriate veterinary supervision.

2.3 Identification of Na_v homologues

To identify *L. salmonis* Na_v homologues, BLASTn searches were performed on the *L. salmonis salmonis* genome assembly (ftp://ftp.ensemblgenomes.org/pub/metazoa/release-36/fasta/lepeophtheirus_salmonis) using the Na_v1 cDNA sequence of the marine copepod *Acartia hudsonica* Pinhey, 1926 [GenBank: KP985762] as the query.[29] Three hits of high homology scores (E-value $<10^{-4}$) were obtained, suggesting that three loci encoding Na_v homologues exist in *L. salmonis* (Supporting information, Table S1). Using ensemble gene models for the three loci, further searches were conducted to identify homologous sequences in other *L. salmonis* genome assemblies (GenBank accession

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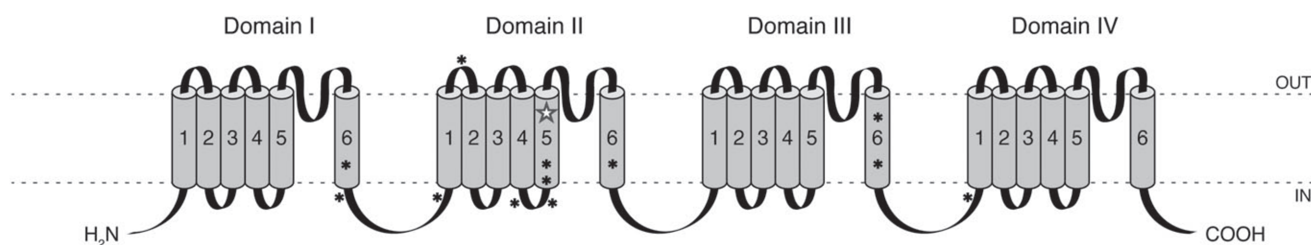

Figure 1. Voltage-gated sodium channel (Na_v1) domain structure. Positions marked with * denote the location of previously found kdr mutations in arthropod species, the effect of which on channel pyrethroid susceptibility was confirmed in *Xenopus* oocytes expression studies.[2] The white star indicates the position of the non-synonymous single nucleotide polymorphism a3041g in *LsNav1.3*, corresponding to the point mutation I936V in the predicted amino acid sequence of the channel (numbering according to *Musca domestica* Vssc1).

numbers GCA_001005205.1 and GCA_000181255.2) and a transcriptome assembly of the species (EBI ENA reference ERS237607) (Supporting information, Table S1).[30]

2.4 Phylogenetic analyses

Phylogenetic analyses of *L. salmonis* Na_v protein sequences took into account sodium channels from a number of arthropods, including several crustaceans and the planktonic copepod *A. hudsonica*.[29] Na_v channels are formed by four homologous domains (I-IV) responsible for channel opening, ion selectivity and voltage sensing linked by highly variable intracellular loops (Fig. 1).[31] Full and partial protein sequences of 21 invertebrate species were aligned using the programme Clustal Omega[32] and then subjected to phylogenetic analysis using the RAxML package.[33] For the planktonic copepod *Calanus finmarchicus* (Gunnerus, 1770), one full-length CDS was included (GenBank: GAXK01037395.1) and the partial sequences of two paralogous Na_v1 genes,[34] since full-length sequences were not available. The phylogenetic trees were constructed from the amino acid sequences of domain I using a maximum likelihood method implementing the WAT model with 1000 bootstrapping iterations.

2.5 *L. salmonis* cDNA synthesis

To isolate total RNA, individual parasites (adult males or preadult II females) were homogenized in TRI Reagent (Sigma-Aldrich, UK) using a Mini-Beadbeater-24 (BioSpec Products). RNA extraction followed the TRI Reagent manufacturer's instructions. The quantity and integrity of isolated RNA was determined by agarose gel electrophoresis and spectrophotometry (Nanodrop ND-1000, Thermo Scientific). Two micrograms of total RNA were reverse transcribed using the Verso cDNA synthesis kit (Thermo Scientific, UK), using 7.5 µM random hexamers and 2.5 µM oligo(dT). The resulting cDNA was diluted 20-fold with nuclease-free water and stored at −70 °C prior to further use.

2.6 Na_v amplification and sequencing

To confirm the sequences of the three *L. salmonis* Na_v1 homologues identified *in silico,* and to conduct sequence comparisons between pyrethroid susceptible and resistant parasites, channel cDNAs were amplified from *L. salmonis* strains IoA-00 and IoA-02 by RT-PCR. Strain IoA-00 has been consistently deltamethrin-susceptible in all bioassays conducted between 2011 and 2015 (*i.e.*, 100% of observed effect after exposure to 1 µg L^{-1} deltamethrin, data not shown), thus parasites from this strain were used without selection. Strain IoA-02 parasites were variable in their responses at deltamethrin levels greater than 10 µg L^{-1}. For the analysis of channel sequences, highly resistant IoA-02 *L. salmonis* were used, which had failed to show behavioural effects after exposures to 40 µg L^{-1} deltamethrin in bioassays (see below). In both strains, three adult male and three preadult-II female parasites were subjected to sequence analysis, but due to failure of some sequencing reactions, data are only available for five parasites per strain for some channel regions. Specific nested PCR oligonucleotide primers designed to target the *L. salmonis* sodium channels were used to amplify domains DI, DII and DIII of each Na_v1 (Supporting information, Table S2).[2] PCRs were performed using 1 µL of diluted cDNA synthesised as described above with 0.3 µM of each oligonucleotide, 5 mM of each dNTP and 5 units of Takara LA Taq Hot Start polymerase (Takara Bio, USA) in a total volume of 50 µL. Reactions were run in a Mastercycler RealPlex (Biometra, UK). PCR conditions consisted of a denaturing step at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at the specified temperature (Additional file 2, Table S2) and extension at 72 °C for 1 min kb^{-1} expected amplicon size. PCR products were purified (QIAquick PCR Purification Kit, QIAGEN, USA) and sequenced using capillary sequencing technology (Macrogen, Netherlands) and appropriate primers to obtain sequences covering sequences encoding S1 to S6 of domains I, II and III (Supporting information, Table S2). Each sequence generated was manually assessed and trimmed, removing low quality regions. Subsequently, sequences of individual parasites were aligned against the reference IoA-00 consensus sequences using BioEdit (v7.05) to identify nucleotide polymorphisms.

2.7 Genotyping of single nucleotide alleles

For selected single nucleotides (SNPs), genotypes of parasites of four strains (IoA-00, IoA-01, IoA-02, IoA-03; eight males and eight females per strain) were determined through allele-specific PCR assays using universal fluorescence energy transfer (FRET) probes.[35] Genomic DNA was extracted from *L. salmonis* specimens conserved in absolute ethanol using the method of Blanquer,[36] with details having been reported elsewhere.[26] Each SNP assay involved one common primer and two allele specific primers (Supporting information, Table S2). Reaction mixtures (final volume 10 µL) contained 25 ng gDNA (lacking in no-template controls), 166 nM of each allele specific primer, 276 nM of the common primer and 1X KASP ® master mix (LGC Genomics, UK). Assays were run in 96-well plates in a Quantica PCR thermocycler (Bibby Scientific, UK). The PCR programme comprised an initial denaturation / activation step (94 °C for 15 min), 10 cycles of a two-step touchdown programme (denaturation at 94 °C for 20 s, annealing at 65–57 °C for 60 s, with a decrease in annealing temperature of 0.6 °C per cycle) and 33 cycles of a regular two-step programme (94 °C for 20 s, 57 °C for 60 s). Genotypes of individuals were

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derived based on the observed relative strength of signals of the two allele-specific fluorophores FAM and HEX.

2.8 Deltamethrin exposures

The effects of deltamethrin exposures on Na_v transcript expression were tested in salmon lice of strains IoA-00 (drug-susceptible) and IoA-02 (pyrethroid resistant). Experimental exposures of *L. salmonis* were at two levels, 0.05 μg L^{-1} deltamethrin and 2 μg L^{-1} deltamethrin. Control groups received seawater containing 0.05% (v/v) vehicle PEG_{300} (polyethylene glycol, $M_n = 300$). Parasites were collected from host fish, randomly allocated to 300 mL crystallizing dishes containing 100 mL of seawater at 12 °C and incubated for 1 to 3 h before the start of exposures. A stock solution of 0.5 mg mL^{-1} deltamethrin (Pestanal ® analytical standard, Sigma-Aldrich, UK) was prepared in PEG_{300} (polyethylene glycol, $M_n = 300$) and further diluted in seawater. Duplicate dishes, each containing five adult males and five preadult II females, were used for deltamethrin and control treatments. After 30 min of exposure at 12 °C, the exposure solution was carefully decanted and the parasites were rinsed twice with seawater before being transferred to plastic Petri dishes holding fresh seawater. Animals were allowed to recover for 24 h in a 12 °C incubator before their attachment and motility behaviour was rated. Behavioural responses were evaluated blind by an observer unaware of strain affiliation and exposure history. Rating was according to viability criteria 'live' (normal behaviour), 'weak', 'moribund' and 'dead', as defined in an earlier study[37] that slightly modified earlier definitions.[38,39] Parasites were considered unaffected when rated 'live' or 'weak' and affected when rated 'moribund' or 'dead'. All IoA-00 parasites exposed to 2 μg L^{-1} deltamethrin for 30 min followed by 24 h of incubation in seawater were rated 'dead'. In all other groups, maximally 10% of parasites were deemed 'affected' (data not shown). Only individuals deemed unaffected were collected for RNA extraction and subsequent determination of transcript abundance.

2.9 Quantitative RT-PCR of *L. salmonis* Na_v homologues

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to determine the abundance of $Na_v1.1$, $Na_v1.2$ and $Na_v1.3$ transcripts. Oligonucleotide primers for target and reference genes (ribosomal subunit 40S, *40S*; elongation factor 1-alpha, *ef1a* and hypoxanthine-guanine phosphoribosyltransferase, *hgprt*) (Additional file 2, Table S2)[40] were used at 300 μM with 1/160 of the cDNA synthesis reaction (2.5 μL of a 1:20 dilution) and 5 μL of SYBR-green qPCR mix (ABgene, UK) in a total volume of 10 μL. Reactions were run in a Mastercycler RealPlex[2] (Eppendorf, UK) ($n = 6$). Amplifications were carried out including negative controls containing no cDNA (NTC, no template control) and controls omitting reverse transcriptase enzyme (-RT) to ascertain the absence of DNA contamination. Thermal cycle and melting curves were performed as described previously.[41] The size of PCR products was checked by agarose gel electrophoresis along with appropriate markers and the correct identity of amplicons was confirmed by sequencing a random subset of samples. Relative quantification of transcript expression was achieved by including on each PCR plate a parallel set of reactions containing serial dilutions of a pool of all experimental cDNA samples, allowing to derive for each sample the estimated relative copy number of the transcript of interest, corrected for the efficiency of the reaction. The normalized expression values were generated by the ΔΔCt method[42] and the results expressed as mean normalized ratios (±SE) between the RUs of target genes and a reference gene index calculated from the geometric mean of the threshold cycles of the three most stable reference genes (*i.e. 40S*, *ef1a* and *hgprt*) (Supporting information, Table S3).[43]

2.10 Data analysis and statistical tests

Transcript expression was presented as the relative expression ratio of each gene (relative units, RUs). Effects of sex/stage and parasite strain on transcript expression were assessed by two-way ANOVA for each of the sodium channels. The effects of deltamethrin exposure on sodium channel transcription of parasites of the same sex/stage and strain were then assessed by one-way ANOVA, followed by post-hoc comparisons to the control group using Dunnett's test. When performing one-way ANOVAs, a Bonferroni correction was applied to significance levels to keep the experiment-wise type I error rate within the overall alpha level. Channel sequences, obtained by Sanger sequencing of RT-PCR products, were compared between *L. salmonis* strains IoA-00 and IoA-02 by performing the Freeman–Halton extension of the Fisher exact probability test[44] for a 2 × 3 table for each polymorphic nucleotide site, *i.e.* comparing the frequencies of three possible outcomes for each individual parasite (sequence variant 1, sequence variant 2, or double peak) between the two parasite strains tested. After genotyping parasites for SNP loci, allele frequencies were compared among strains using Fisher's method as implemented in the program genepop version 4.2.[45] The significance level was $P < 0.05$ for all statistical tests performed.

3 RESULTS

3.1 Na_v1 homologues in *L. salmonis*

Genomic screening of voltage-gated sodium channels identified three *L. salmonis* Na_v1 homologues (Supporting information, Table S1), referred to in this study as $LsNa_v1.1$, $LsNa_v1.2$ and $LsNa_v1.3$, following to the accepted nomenclature for Na_v channels.[11] Transcript sequences of the three channels identified in a multi-stage transcriptome *of L. salmonis*[30] yielded cDNA sequences covering the whole open reading frame (ORF) for $LsNa_v1.1$ and $LsNa_v1.3$ and a partial sequence for $LsNa_v1.2$ (Supporting information, Table S1). The deduced amino acid sequences of the identified channels contain typical features present in all Na_v1,[2,31] such as four homologous domains (I-IV), the sodium sensing amino acids 'DEKA', and the inactivation gate motif ('IFM' in rat $Na_v1.2$; 'MFM' or 'AFM' in *L. salmonis* Na_v1 homologues) (Supporting information, Fig. S1).

3.2 Phylogenetic analysis

Phylogenetic analyses were performed on sequences of conserved domain I of arthropod Na_v1 channels (Fig. 2). In the obtained tree, sequences cluster together according to taxonomic groups, such as insects, malacostracans, copepods and arachnids. Copepod homologues formed two separate clusters. $LsNa_v1.1$ grouped closely with the sequence of the planktonic copepod *A. hudsonica* and one of three *C. finmarchicus* sodium channels, whereas $LsNa_v1.2$ and $LsNa_v1.3$ grouped more distantly together with two remaining *C. finmarchicus* homologues (Fig. 2).

3.3 Channel nucleotide sequences in resistant and susceptible parasites

Na_v1 channels comprise four internally homologous domains (I-IV) that each contain six α-helical transmembrane segments (S1–S6) connected by intracellular loops.[31] Kdr type resistance shows a

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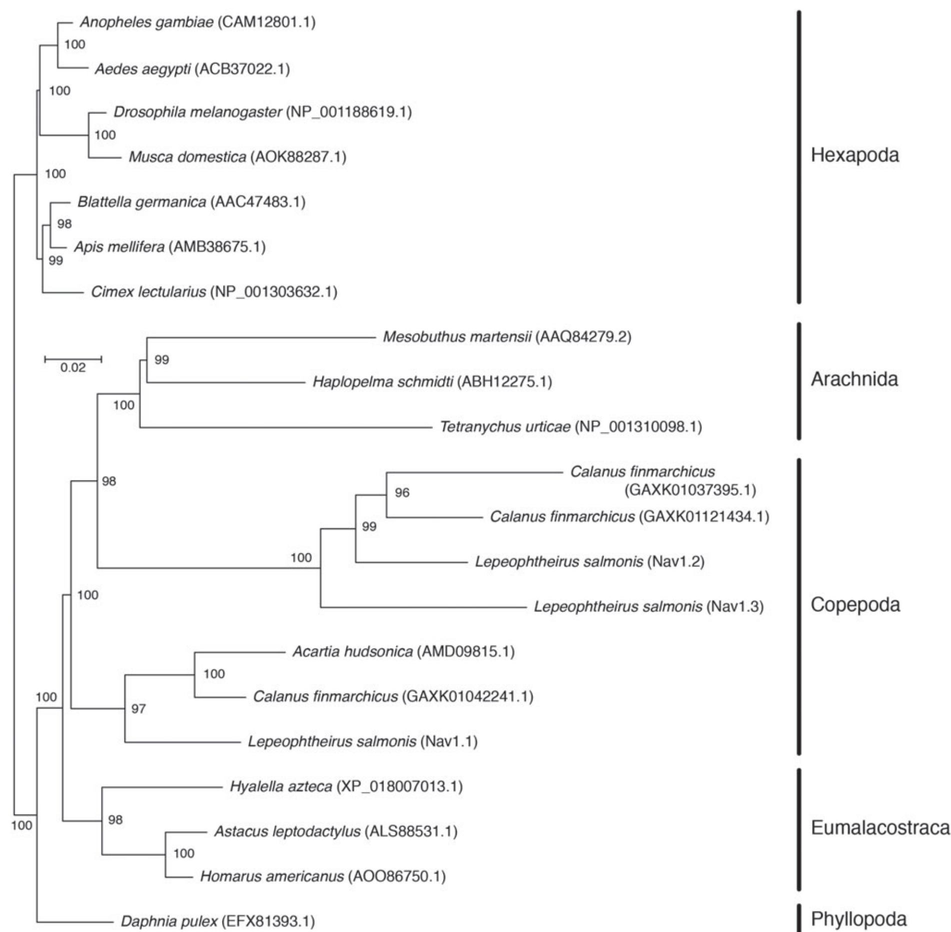

Figure 2. Phylogenetic analysis of voltage-gated sodium channels Na_v1 in *L. salmonis*. The tree was constructed based on the amino acid sequences of domain I of Na_v1 paralogues identified in a salmon louse transcriptome[29] subjected to a maximum likelihood analysis using RAxML.[33] For phylogenetic reconstruction, the WAG substitution model of rate heterogeneity among sites were implemented. Numbers at the basal nodes represent the frequencies with which the presented tree topology was obtained after bootstrapping (1000 iterations). The scale bar represents 0.2 amino-acid substitutions per site.

recessive mode of inheritance in other arthropods[31] and is associated with mutations in specific regions of domains I-III, namely segments S5 and S6 and the linker helix connecting S4 and S5. For channels *Na_v1.1*, *Na_v1.2* and *Na_v1.3*, domains I to III were amplified by RT-PCR in both susceptible (IoA-00) and pyrethroid-resistant (IoA-02) salmon lice ($n \geq 5$ animals per strain) and sequenced. While a number of sequence polymorphisms were identified for genes *LsNa_v1.1* and *LsNa_v1.2*, their frequencies did not differ significantly between IoA-00 and IoA-02 salmon lice (Supporting information, Tables S4, S5). In contrast, sequences at two polymorphic sites in *LsNa_v1.3* were significantly affected by parasite strain origin (Supporting information, Table S6). The nucleotide polymorphism a3947c corresponds to a missense mutation, causing an amino acid change located in the intracellular loop between DII and DIII, while g4465a represents a synonymous mutation at a site in IIIS4 of the channel (counts according to HACA01028321.1). Moreover, strain differences were apparent but not significant ($P = 0.061$) for a non-synonymous mutation at a site in IIS5 of *LsNa_v1.3* (a3041g, Supporting information, Table S6).

For *LsNa_v1.3* mutations that showed a trend towards sequence differences between IoA-00 and IoA-02 ($P < 0.1$, Supporting information, Table S6), potential association with deltamethrin resistance was further investigated by assessing the genotypes of a greater number of parasites in four strains, using allele-specific PCR assays. Deltamethrin susceptible strains IoA-00 and IoA-01 as well as deltamethrin resistant strains IoA-02 and IoA-03 were considered at $n = 14$ to 16 parasites per strain (Table 1). For the SNP marker a3041g, allele frequencies differed significantly between all possible pairings of deltamethrin resistant and susceptible strains, and between resistant strains IoA-02 and IoA-03 (Table 2). Allele frequencies at SNP loci a3947c and g4465a did not significantly differ among strains IoA-00, IoA-01 and IoA-03, but were significantly different in strain IoA-02 as compared to the other studied strains (Table 2).

3.4 Transcript expression of *L. salmonis* voltage-gated Na channels

L. salmonis are sexually dimorphic, with the development of parasites from the hatching of eggs to reaching the adult stage taking longer to complete in females than males. In synchronized cohorts of developing parasites, preadult II females and adult males appear at the about the same time and are similar in size. For this reason, these stages are routinely used for bioassays in our laboratory. In order to make findings of this study comparable to bioassay data, preadult II females and adult male sea lice, obtained from synchronized cohorts of strains IoA-00 and IoA-02, were used in studies of transcript expression of paralogous *Na_v* genes (Fig. 3). For each Na_v

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Table 1. Genotyping of different *L. salmonis* strains at SNP loci within *LsNa$_v$1.3*. Individuals from deltamethrin (DM) susceptible (S) and resistant (S) strains were subjected to allele specific PCR genotyping. Per strain, 6–8 animals of each sex were tested. Data were pooled within strains as no significant sex-specific allelic or genotypic differentiation was found. Genotype frequencies in the different strains showed no significant deviation from Hardy–Weinberg equilibrium

SNP locus	Strain	DM susceptibility	*n*	Genotypes (%) gg	ga	aa	Frequency allele 1	Frequency allele 2
a3041g	IoA-00	S	16	0.00	0.00	100.00	0.000	1.000
	IoA-01	S	16	0.00	0.00	100.00	0.000	1.000
	IoA-02	R	15	60.00	40.00	0.00	0.800	0.200
	IoA-03	R	14	21.43	28.57	50.00	0.357	0.643
a3947c	IoA-00	S	16	6.25	62.50	31.25	0.375	0.625
	IoA-01	S	16	6.25	43.75	50.00	0.281	0.719
	IoA-02	R	15	0.00	0.00	100.00	0.000	1.000
	IoA-03	R	14	7.14	14.29	78.57	0.143	0.857
a4456g	IoA-00	S	16	31.25	62.50	6.25	0.625	0.375
	IoA-01	S	16	50.00	43.75	6.25	0.719	0.281
	IoA-02	R	15	100.00	0.00	0.00	1.000	0.000
	IoA-03	R	14	78.57	14.29	7.14	0857	0.143

Table 2. Pairwise allelic differentiation between *L. salmonis* strains at three SNP loci. The table shows *P*-values of pairwise comparisons of allele frequencies between deltamethrin susceptible (S) and resistant (R) strains using the exact G-test, based on the data shown in Table 1. Allele frequencies are regarded significantly different between strains when the *P*-value is lower than 0.05 (given in bold print)

SNP locus	Strains	IoA-01 (S)	IoA-02 (R)	IoA-03 (R)
a3041g	IoA-00 (S)	1.00000	**<0.0001**	**<0.0001**
	IoA-01 (S)		**<0.0001**	**0.00014**
	IoA-02 (R)			**0.00127**
a3947c	IoA-00 (S)	0.58604	**0.00026**	0.15480
	IoA-01 (S)		**0.00167**	0.38541
	IoA-02 (R)			**0.02016**
a4456g	IoA-00 (S)	0.79582	**0.00012**	0.08039
	IoA-01 (S)		**0.00029**	0.13766
	IoA-02 (R)			**0.04910**

channel, two-way ANOVA was used to assess whether transcription differed between the IoA-00 and IoA-02 strains and between preadult II females and adult males. Transcript abundance was not affected by strain origin for any of the studied *Na$_v$* genes but differed significantly between adult males and preadult II females for all three channels ($P < 0.001$). Moreover, there was a significant interaction between strain origin and sex/stage effects on the transcription of *Na$_v$*1.2 and *Na$_v$*1.3 ($P < 0.05$). When *Na$_v$* gene transcription was studied after a 30 min exposure to deltamethrin followed by 24 h recovery, deltamethrin (0.05 μg L^{-1}) significantly decreased *Na$_v$1.3* transcription ($P < 0.05$) in IoA-00 adult males (Fig. 3).

4 DISCUSSION

The present study characterized voltage-gated sodium channels in the fish parasite *L. salmonis*, in which pyrethroid resistance is common.[20] The results revealed the existence of three voltage-gated sodium channel paralogues in *L. salmonis* that were named *LsNa$_v$1.1*, *LsNa$_v$1.2* and *LsNa$_v$1.3* according to the accepted nomenclature.[11,46] The predicted salmon louse Na$_v$1 polypeptides are approximately 40% identical to vertebrate Na$_v$1 subunits and comprise four highly conserved homologous domains (I-IV), paralleling the architecture of all known voltage-gated sodium channels.[1,2] Each domain contains six hydrophobic transmembrane segments (S1–S6) including the voltage sensor in the S4 segments, characterized by positively charged arginine amino acid residues. A re-entrant loop between S5 and S6 embedded into the transmembrane region of the channels was identified, known to form the Na$^+$ selective filter (SF) constituted by four amino acids (Asp, Glu, Lys and Ala, [DEKA]).[47] The presence of a lysine (K) in the third position has proven crucial for Na$^+$ permeability.[48]

Kdr-type resistance of arthropods against pyrethroids and DDT results from point mutations of Na$_v$1 leading to amino acid changes of the channel.[2,49–51] Since the initial identification of Na$_v$1 mutations in *kdr* and *super-kdr* strains of the housefly,[52,53] many kdr-type mutations have been reported from resistant isolates of different arthropod species,[2] with key mutations having evolved independently in different arthropod species.[5] The comparative functional characterization of wild type Na$_v$1 and selected channels with kdr mutations following recombinant expression in *Xenopus* oocytes revealed that many kdr-type mutations decrease the pyrethroid affinity of Na$_v$1.[2] Moreover, functional studies of Na$_v$1 mutants showed that some single amino acid substitutions have synergistic effects when present in combination.[2] Interestingly, kdr mutations cluster in particular channel regions, such as the linker between S4 and S5 (L45) and S5 of DI, L45, S5 and S6 of DII, and S6 of DIII,[2] suggesting Na$_v$1 may possess discrete pyrethroid binding sites. Potential Na$_v$1-ligand interactions were assessed through the construction of homology models of insect Na$_v$1 based on available crystal structures of bacterial sodium and mammalian potassium channels.[54,55] This approach led to the prediction of two pyrethroid binding sites, one of which involves L45 and S5 of DII and S6 of DIII, while the second site is composed of L45 and S5 of DI and S6 of DII.[56,57]

In order to identify kdr in the present study, *L. salmonis Na$_v$1* sequences were amplified from a restricted number of individuals ($n = 5$–6) of two strains (IoA-00, drug susceptible; IoA-02, highly pyrethroid resistant) and sequenced. Using this approach three candidate nucleotide polymorphisms potentially associated

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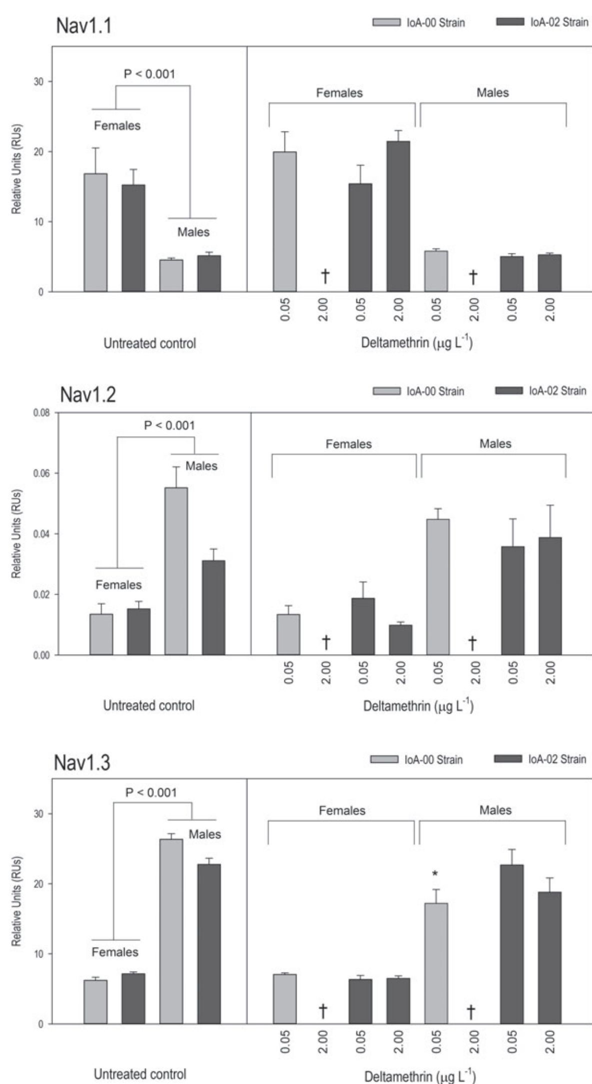

Figure 3. Expression of voltage-gated sodium channels Na_v1 in *L. salmonis* preadult II females and adult males. Transcript expression was expressed as relative units (RUs) calculated from the mean normalised ratios ($n = 6$, ±SE) between the estimated copy numbers of target genes and the estimated copy numbers of the reference genes. Columns represent the normalised expression values determined in parasites of different strains (IoA-00, drug-susceptible; IoA-02 pyrethroid-resistant) and instar after 30 min of treatment with the indicated level of deltamethrin or solvent vehicle PEG300 (controls) and 24 h of recovery. In treatments labelled with a dagger (†), all parasites were rated moribund or dead and therefore excluded from analyses. Asterisks (*) indicate a significant treatment effect on channel transcription when compared to control animals of the same strains and sex/instar (One-way ANOVA and Dunnett's test; $P < 0.05$ after Bonferroni correction).

to resistance were identified in *LsNa$_v$1.3* (a3041g, a3947c and a4456g), while no potential kdr mutations were found in *LsNa$_v$1.1* and *LsNa$_v$1.2*. The *LsNa$_v$1.3* candidate markers were then further assessed by genotyping a larger number of individuals ($n = 14-16$) in the above and two additional strains. Data obtained with the non-synonymous a3041g mutation supported a putative association between deltamethrin resistance and the expression of the 3041 g allele, which was present in the deltamethrin resistant strains studied, IoA-02 and IoA-03, but not detected in the deltamethrin susceptible strains IoA-00 and IoA-01. In contrast, genotyping further individuals and taking into account further parasite strains failed to confirm an association of single nucleotide polymorphisms a3947c and a4456g with deltamethrin resistance.

The non-synonymous a3041g mutation results in the substitution of an isoleucine by a valine at amino acid position 1014 of LsNa$_v$1.3 (numbering according to predicted amino acid sequence of GenBank accession number HACA01028321.1). When numbered according to *Musca domestica* Vssc1 (GenBank accession number: AAB47604), this mutation corresponds to I936V located in S5 of DII (Figs 1, S1), a conserved channel region contributing to one of the two predicted pyrethroid binding sites and known to contain kdr mutations.[2] Interestingly, a homologous mutation has been identified in the *Na$_v$1* sequence of pyrethroid resistant corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (I951V, also corresponding to I936V of *M. domestica* Vssc1), where it was found in the cDNA but not gDNA sequence, suggesting its introduction by RNA editing.[58] In contrast, the a3041g mutation was apparent in *L. salmonis* in both cDNA and gDNA sequences (this study). Although isoleucine and valine are amino acids with similar physiochemical properties, the substitution within one of the two predicted pyrethroid-binding sites of the channel corresponds to a position of Na$_v$1 where valine is the residue found in vertebrates, while isoleucin is found in the wild-type channel of arthropods. O'Reilly *et al.*[57] suggested that this difference in sequence could contribute to species-dependent binding affinity of pyrethroids to Na$_V$1, and subsequently differences in toxicity. Furthermore, the isoleucine to valine mutation in the *Na$_V$1* gene in *Drosophila melanogaster* was confirmed to cause decreased pyrethroid sensitivity after cloning into in the *Xenopus* oocyte system and subsequent electrophysiological analysis.[59]

An earlier study assessed Na$_v$1.1 sequences in *L. salmonis* sampled from different salmon farming sites.[23] A non-synonymous mutation in *LsNa$_v$1.1*, which leads to a glutamine to arginine change at a position corresponding to amino acid 945 of the *Musca domestica* Na$_v$1, was present in 10% to 67% of *L. salmonis* of populations collected at fish farms where decreased efficacy of pyrethroids had been reported, and absent in parasite populations from sites with no reports of pyrethroid treatment failures.[23] While the Q945R mutation maps to a Na$_v$1 region harbouring known kdr mutations, no direct evidence exists that the mutation affects Na$_v$1 affinity to pyrethroids.[23] In a later study on individually selected pyrethroid resistant salmon lice from four locations in Norway,[60] neither this, nor other pyrethroid-associated mutations were found in *LsNa$_v$1.1* from adult females (Supporting information, Table S7).

A recent study demonstrated a sex-biased pattern of inheritance of pyrethroid resistance in *L. salmonis*, studying strains from Scottish sites also investigated in the present study.[26] In reciprocal crosses between pyrethroid resistant IoA-02 salmon lice and susceptible IoA-00 parasites, the F1 and F2 progeny of all families derived from a deltamethrin susceptible P0 male and a deltamethrin resistant P0 female were resistant.[26] In contrast, in families derived from crosses of the inverse orientation, F1 animals were susceptible and, depending on the family, 0–20% of F2 parasites were resistant. Maternal inheritance of deltamethrin resistance in *L. salmonis* has also been demonstrated in a separate study on Norwegian salmon lice strains.[25] In the Scottish study, deltamethrin resistant strains isolated from different regions of Scotland showed nearly identical mitochondrial haplotypes.[26] The results suggested a major contribution of mitochondrial genetic factors to pyrethroid resistance in *L. salmonis;* however the presence of the 20% resistant F2 parasites in some families initiated from an IoA-02 male and an IoA-00 female suggests that

nuclear genetic determinants may be responsible for the additional observed resistance. Kdr resistance in other arthropods is a recessive trait[4] and such a mode of inheritance would be consistent with the observed resistance phenotype in the F2 crosses.[26]

In mammals, multiple Na_v1 paralogues have arisen from gene duplications and chromosomal rearrangements, and differ in channel gating kinetics, ontogeny and tissue expression profiles, with certain isoforms primarily involved in the nervous response while others are majorly expressed in the skeletal muscle or the heart.[31,46] In contrast, in insects multiple Na_v isoforms are typically produced through mechanisms of alternative splicing.[31,34,61] For instance, in *Drosophila*, one Na_v1 gene exhibits multiple alternative exons that can be used to produce 29 splice types, and although no pharmacological tests have been performed to determine specific drug and toxin affinity, the functional characterization revealed clear differences in the channel kinetics.[61]

Relatively little is known about Na_v1 genes in crustaceans. A single Na_v1 gene has been found in the genome of the cladoceran *Daphnia*.[34] In contrast, the copepod *C. finmarchicus* possesses a diverse Na_v1 subfamily with at least three isoforms,[34,62] paralleling the findings obtained with *L. salmonis* in the present study. Planktonic free-living copepods, believed to be the most abundant metazoans in the oceans,[63] are integral to marine food webs.[64] Thus, multiplicity of Na_v channel function could be related to the adaptation of copepods to a variety of stressors, for example, exposure to algal toxins targeting Na_v1.[62] The presence of different Na_v1 homologues in *L. salmonis* could further be related to subfunctionalization, such as different types of neurons expressing different Na_v1 paralogues. The fact that a kdr mutation was observed in *LsNa$_v$1.3*, but not *LsNa$_v$1.1* and *LsNa$_v$1.2*, could point to a greater importance of *LsNa$_v$1.3* for survival during the host-attached phase of the life cycle. More research is needed to resolve functions of Na_v1 paralogues in *L. salmonis*.

In the present study, relative expression of *L. salmonis* Na_v1 transcripts showed a similar pattern of transcription for *LsNa$_v$1.2* and *LsNa$_v$1.3*, for both of which transcript expression was low in preadult II females and high in adult males. In contrast, the *LsNa$_v$1.1* transcript was more abundant in preadult II females as compared to adult males of the same age. In *L. salmonis* male reproductive behaviours include mate searching and testing, formation of precopulatory complexes with preadult-II females and mate guarding.[65] Copulation with the guarding male occurs typically directly after the moult of the female to the final adult stage, and involves the male cementing a pair of spermatophores onto the female's genital complex, blocking further insemination.[66] However, using microsatellite markers, multiple paternity of offspring of individual females was demonstrated, providing evidence for polyandry in *L. salmonis*.[67] In view of the marked contrasts in reproductive behaviours between the sexes, it may be speculated that sexually differential Na_v1 transcription could reflect sex specific neuronal adaptations.

5 CONCLUSION

Findings of the present study indicate that a kdr-mutation in *LsNa$_V$ 1.3* may contribute to deltamethrin resistance in *L. salmonis*.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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