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

Running title: Kdr mutations in *L. 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 characterised 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*.

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 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 & 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 & 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 characterised 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 2012, 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 μg L^{-1} (0.23 – 0.36 μg L^{-1}), IoA-01: 0.36 μg L^{-1} (0.26 – 0.46 μg L^{-1}), IoA-02: 40.1 μg L^{-1} (22.1 – 158.9 μg L^{-1}), IoA-03: >2.0 μg 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 euthanised 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 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 (Figure 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 homogenised 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), employing 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 sec, 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 (SNP) alleles

For selected SNPs, genotypes of parasites of four strains (IoA-00, IoA-01, IoA-02, IoA-03; 8 males and 8 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 (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 (94ºC for 15 min), ten cycles of a two-step touchdown programme (denaturation at 94ºC for 20 sec, annealing at 65 - 57ºC for 60 sec, with a decrease in annealing temperature of 0.6ºC per cycle) and 33 cycles of a regular two-step programme (94ºC for 20 sec, 57ºC for 60 sec). Genotypes of individuals

were 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 crystallising 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 5 adult males and 5 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 normalised expression values were generated by the ΔΔCt method [42] and the results expressed as mean normalised 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 x 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$_v$1.1* and *LsNa$_v$1.3* and a partial sequence for *LsNa$_v$1.2* (Supporting information, Table S1). The deduced amino acid sequences of the identified channels contain typical features present in all Na$_v$1 [2,31], such as four homologous domains (I-IV), the sodium sensing amino acids "DEKA", and the inactivation gate motif ("IFM" in rat Na$_v$1.2; "MFM" or "AFM" in *L. salmonis* Na$_v$1 homologues) (Supporting information, Figure S1).

3.2 Phylogenetic analysis

Phylogenetic analyses were performed on sequences of conserved domain I of arthropod Na$_v$1 channels (Figure 2). In the obtained tree, sequences cluster together according to taxonomic groups, such as insects, malacostracans, copepods and arachnids. Copepod homologues formed two separate clusters. *LsNa$_v$1.1* grouped closely with the sequence of the planktonic copepod *A. hudsonica* and one of three *C. finmarchicus* sodium channels, whereas *LsNa$_v$1.2* and *LsNa$_v$1.3* grouped more distantly together with two remaining *C. finmarchicus* homologues (Figure 2).

3.3 Channel nucleotide sequences in resistant and susceptible parasites

Na$_v$1 channels comprise four internally homologous domains (I-IV) that each contain six α-helical transmembrane segments (S1-S6) connected by intracellular loops [31]. Kdr type resistance shows a recessive mode of inheritance in other arthropods [31] and is associated with mutations in specific regions of domains I-III, namely segments S5 and S6 and the linker helix connecting S4 and S5. For channels *Na$_v$1.1, Na$_v$1.2* and *Na$_v$1.3*, domains I to III were amplified by RT-PCR in both susceptible (IoA-00) and pyrethroid-resistant (IoA-02) salmon lice (n ≥ 5 animals per strain) and sequenced. While a number of sequence polymorphisms were identified for genes *LsNa$_v$1.1* and *LsNa$_v$1.2*, their frequencies did not differ significantly between IoA-00 and IoA-02 salmon lice (Supporting information, Tables S4-5). In contrast, sequences at two polymorphic sites in *LsNa$_v$1.3* were significantly affected by parasite strain origin (Supporting information, Table S6). The nucleotide polymorphism a3947c corresponds to a missense mutation, causing an amino acid change located in the intracellular loop between DII and DIII, while g4465a represents a synonymous mutation at a site in IIIS4 of the channel (counts according to HACA01028321.1). Moreover, strain differences were apparent but not significant (P=0.061) for a non-synonymous mutation at a site in IIS5 of *LsNa$_v$1.3* (a3041g, Table S6).

For *LsNa$_v$1.3* mutations that showed a trend towards sequence differences between IoA-00 and IoA-02 (P<0.1, 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 synchronised 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 synchronised cohorts of strains IoA-00 and IoA-02, were used in studies of transcript expression of paralogous Na_v genes (Figure 3). For each Na_v 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 $Na_v1.2$ and $Na_v1.3$ ($P < 0.05$). 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 $Na_v1.3$ transcription ($P < 0.05$) in IoA-00 adult males (Figure 3).

4 Discussion

The present study characterised 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$1 polypeptides are ~40% identical to vertebrate Na$_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, characterised 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$1 mutations in *kdr* and *super-kdr* strains of the housefly [52,53], many kdr-type mutations have been reported from resistant isolates of different arthropod species [2], with key mutations having evolved independently in different arthropod species [54]. The comparative functional characterisation of wild type Na$_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 [55,56]. 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 [57,58].

In order to identify kdr in the present study, *L. salmonis Na$_v$1* sequences were amplified from a restricted number of individuals (n=5-6) of two strains (IoA-00, drug susceptible; IoA-02, highly pyrethroid resistant) and sequenced. Using this approach three candidate nucleotide polymorphisms potentially associated to resistance were identified in *LsNa$_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 3041g 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$1.3 (numbering according to predicted amino acid sequence of GenBank accession number HACA01028321.1). When numbered according to *Musca domestica* Vssc1 (GenBank accession number: AAB47604), this mutation corresponds to I936V located in S5 of DII (Figure 1, Figure S1), a conserved channel region contributing to one of the two predicted pyrethroid binding sites and known to contain kdr mutations[2]. Interestingly, a homologous mutation has been identified in the *Na$_v$1* sequence of pyrethroid resistant corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (I951V, also corresponding to I936V of *M. domestica* Vssc1), where it was found in the cDNA but not gDNA sequence, suggesting its introduction by RNA editing [59]. In contrast, the a3041g mutation was apparent in *L. salmonis* in both cDNA and gDNA sequences (this study). Although isoleucine and valine are amino acids with similar physiochemical properties, the substitution within one of the two predicted pyrethroid-binding sites of the channel corresponds to a position of Na$_v$1 where valine is the residue found in vertebrates, while isoleucin is found in the wild-type channel of arthropods. O'Reilly et al.[58] suggested that this difference in sequence could contribute to species-dependent binding affinity of pyrethroids to Na$_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[60].

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[61], 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$_v$1 paralogues have arisen from gene duplications and chromosomal rearrangements, and differ in channel gating kinetics, ontogeny and tissue expression profiles, with certain isoforms primarily involved in the nervous response while others are majorly expressed in the skeletal muscle or the heart [31,46]. In contrast, in insects multiple Na$_v$ isoforms are typically produced through mechanisms of alternative splicing [31,34,62]. For instance, in *Drosophila*, one *Na$_v$1* 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 characterisation revealed clear differences in the channel kinetics [62].

Relatively little is known about *Na$_v$1* genes in crustaceans. A single *Na$_v$1* gene has been found in the genome of the cladoceran *Daphnia* [34]. In contrast, the copepod *C. finmarchicus* possesses a diverse *Na$_v$1* subfamily with at least three isoforms [34,63], paralleling the findings obtained with *L. salmonis* in the present study. Planktonic free-living copepods, believed to be the most abundant metazoans in the oceans [64], are integral to marine food webs [65]. Thus, multiplicity of Na$_v$ channel function could be related to the adaptation of copepods to a variety of stressors, e.g. exposure to algal toxins targeting Na$_v$1 [63]. The presence of different Na$_v$1 homologues in *L. salmonis* could further be related to subfunctionalisation, such as

different types of neurons expressing different Na_v1 paralogues. The fact that a kdr mutation was observed in *$LsNa_v1.3$*, but not *$LsNa_v1.1$* and *$LsNa_v1.2$*, could point to a greater importance of *$LsNa_v1.3$* 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 *$LsNa_v1.2$* and *$LsNa_v1.3$*, for both of which transcript expression was low in preadult II females and high in adult males. In contrast, the *$LsNa_v1.1$* 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 [66]. 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 [67]. However, using microsatellite markers, multiple paternity of offspring of individual females was demonstrated, providing evidence for polyandry in *L. salmonis* [68]. 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.

Conclusion

Findings of the present study indicate that a kdr-mutation in *$LsNa_V1.3$* may contribute to deltamethrin resistance in *L. salmonis*.

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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.

				Genotypes (%)				
SNP locus	**Strain**	**DM susceptibility**	**N**	**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**

Figure Legends

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).

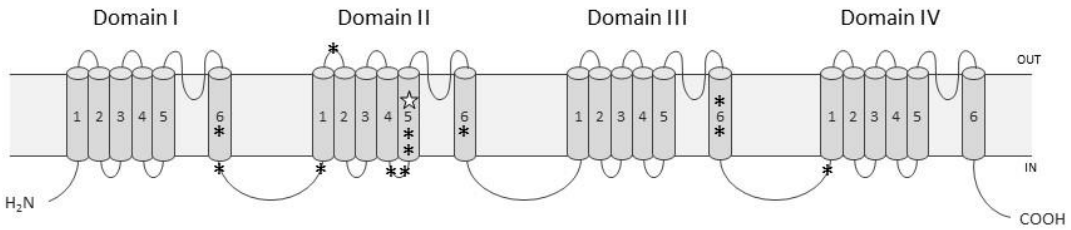

Figure 2. Phylogenetic analysis of voltage-gated sodium channels Na_v1 in *L. salmonis*. The tree was constructed based on the amino acid sequences of domain I of Na_v1 paralogues identified in a salmon louse transcriptome [29] subjected to a maximum likelihood analysis using RAxML [33]. For phylogenetic 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.

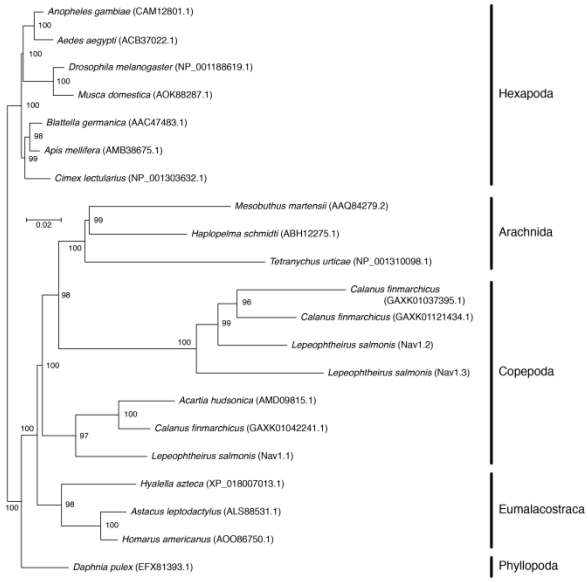

Figure 3. Expression of voltage-gated sodium channels Na_v1 in *L. salmonis* preadult II females and adult males. Transcript expression was expressed as relative units (RUs) calculated from the mean normalised ratios (n=6, ±SE) between the estimated copy numbers of target genes and the estimated copy 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).