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Received: 5 August 2020 Revised: 24 September 2020 Accepted article published: 1 October 2020 Published online in Wiley Online Library: 17 October 2020

(wileyonlinelibrary.com) DOI 10.1002/ps.6120

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

Claudia Tschesche,[a*] Michaël Bekaert,[a] David I Bassett,[a] Chris Mitchell,[b] Ben North,[b] Sally Boyd,[a] Greta Carmona-Antoñanzas,[a,c] James E Bron[a] and Armin Sturm[a]

Abstract

BACKGROUND: The pyrethroid deltamethrin is used to treat infestations of farmed salmon by parasitic salmon lice, *Lepeophtheirus salmonis* (Krøyer). However, the efficacy of deltamethrin for salmon delousing is threatened by resistance development. In terrestrial arthropods, knockdown resistance (*kdr*) mutations of the voltage-gated sodium channel (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 been identified previously. At the same time, deltamethrin resistance of *L. salmonis* has been shown to be inherited maternally and to be associated with mitochondrial DNA (mtDNA) mutations. This 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*.

RESULTS: 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$1.3 I936V showed no association with deltamethrin resistance. By contrast, genotypes at the mtDNA loci A14013G and A9030G were significantly associated with deltamethrin resistance ($P < 0.001$).

CONCLUSION: 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.

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

Keywords: deltamethrin; salmon lice; resistance; sodium channel; mitochondria; knockdown resistance

1 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.[1] 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.[2,3] In 2018, the estimated global costs of sea lice infections to the salmon industry were approximately US $873 million (£700 million),[4] 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* (Krøyer).[5] At salmon production sites, sea lice are controlled by integrated pest management strategies combining non-medicinal approaches, such

* Correspondence to: C Tschesche, Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, UK, E-mail: claudia.tschesche@stir.ac.uk

Funding information
PHARMAQ/Zoetis; United Kingdom Biotechnology and Biological Sciences Research Council, Grant Number: BB/L022923/1; The Marine Alliance for Science and Technology for Scotland funded by the Scottish Funding Council, Grant Number: HR09011; Scottish Salmon Producer's Organisation.

a Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling, UK

b PHARMAQ Ltd., Fordingbridge, UK

c Imegen, Parc Cientific de la Universitat de Valencia, Valencia, Spain

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as mechanical and thermal delousing, physical barriers,[6] and biological control through co-culture with cleaner fish,[7] and medicinal approaches employing a limited range of licensed veterinary medicines.[8] 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.[9] However, in *L. salmonis* populations of the North Atlantic, loss of efficacy has been reported for most available salmon delousing agents.[9–11]

Pyrethroids, which are synthetic analogues of the botanical pyrethrins,[12] are widely used to control insects that are phytophagous, parasitic, or represent vectors for human disease.[13] In 2014, pyrethroids accounted for 17% of global insecticide use.[13] In arthropods, the toxic action of pyrethroids is based on their blocking of the voltage-gated sodium channel (Na_v), which plays an essential role in the initiation and propagation of nerve impulses.[14] In terrestrial arthropods, two main mechanisms of deltamethrin resistance are known: knockdown (*kdr*) resistance by target-site mutations in Na_v,[15,16] and increased detoxification by enhanced expression of metabolic enzymes, such as carboxylesterases, cytochrome P450s (CYP), or glutathione *S*-transferases (GSTs).[17]

In *L. salmonis*, resistance to the pyrethroid deltamethrin is widespread,[11] 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$_v$1.1, LsNa$_v$1.2 and LsNa$_v$1.3), led to the identification of a putative *kdr* mutation in LsNa$_v$1.3,[18] which causes an amino acid change (I936V) in the predicted pyrethroid binding site of the channel and is homologous to a previously described *kdr* mutation in pyrethroid-resistant isolates of the phytophagous moth *Helicoverpa zea* (Boddie).[19,20] On the other hand, crossing experiments in which deltamethrin-resistant *L. salmonis* were interbred with drug-susceptible parasites of the opposite sex to generate multigenerational families revealed a predominantly maternal inheritance of deltamethrin resistance.[21] In particular, all second filial generation (F2) parasites of families derived from crosses between resistant females and susceptible males were resistant, whereas < 20% resistant F2 parasites were observed in the inverse crosses derived from susceptible females and resistant \males, suggesting a role of mitochondrial genes as determinants of deltamethrin resistance.[21] Deltamethrin resistance in independent isolates obtained from different Scottish regions was associated with virtually identical mitochondrial (mtDNA) haplotypes, which contained the mitochondrial SNP A14017G located within the cytochrome *B* (CytB) gene and SNP A9030G located in cytochrome *c* oxidase subunit I (COX1) gene.[21,22] These findings suggested that the mode of action of deltamethrin in *L. salmonis* might involve mitochondrial targets. In support of this hypothesis, deltamethrin has been shown to affect mitochondrial functions such as ATP production[21] and induced apoptosis in skeletal muscle tissues, which have a high mitochondria content.[23] However, efforts to disentangle the role of nuclear and mitochondrial mutations in pyrethroid resistance are complicated by the fact that deltamethrin-resistant *L. salmonis* investigated in previous studies displayed both the putative *kdr* mutation LsNa$_v$1.3 I936V and mtDNA mutations.[18,21]

The aim of this study was to assess potential roles of the LsNa$_v$1.3 I936V mutation as a determinant of deltamethrin resistance in *L. salmonis*. First, *L. salmonis* strains differing in deltamethrin resistance and expression of the LsNa$_v$1.3 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 MATERIALS AND METHODS

2.1 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. This 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.

2.2 *Lepeophtheirus salmonis* strains and husbandry

Laboratory *L. salmonis* strains used in this study have been described in detail elsewhere.[24,25] 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}; IoA-02: 40.1 μg L^{-1}, 22.1–158.9 μg L^{-1}).[21]

Lepeophtheirus 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.[24] In brief, *L. salmonis* were kept on Atlantic salmon (*Salmo salar*, L.) held in circular tanks provided with a continuous supply of seawater 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 naive Atlantic salmon. Infection trials were set up to produce preadult II and adult parasites for bioassays and molecular analyses. Host fish were killed using a UK Home Office approved Schedule 1 method prior to the removal of salmon lice from fish.

2.3 *Lepeophtheirus salmonis* crosses

This study included molecular analyses of archived *L. salmonis* siblings of one family from a previously published crossing experiment (Fig. S1), with the detailed procedures of performing the cross having been reported elsewhere.[21] 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

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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 below).

2.4 *Lepeophtheirus salmonis* field populations

Bioassays and molecular analyses were carried out with *L. salmonis* obtained from Scottish aquaculture sites (Table S1). 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 below). Samples were transported in insulated boxes equipped with cold packs and arrived within 6 h of initial collection.

2.5 *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® (PHARMAQ, 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 five preadult II females and five 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 2000× final concentration solution of the relevant compound to glass crystallising dishes containing 100 mL seawater and parasites. To prepare the 2000× final concentration solutions, etofenprox was solubilised in ethanol, whereas deltamethrin was dissolved in PEG_{300} polyethylene glycol ($M_n = 300$; PEG_{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.[26,27] 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 completion of the 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.[25] Parasites rated as 'live' or 'weak' were considered unaffected, whereas '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 below), 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.

2.6 DNA extraction

Genomic DNA was extracted from individual ethanol-conserved salmon louse specimens using a high-throughput protocol.[28] 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 4000 *g* for 1 min. Crude DNA extracts were stored at −20 °C pending use in genotyping analyses.

2.7 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 *Musca domestica* L. *Vssc1* (GenBank accession number: AAB47604),[18] and *L. salmonis* mtDNA SNPs A14013G (CytB, cds) and A9030G (COX1, cds),[21] with oligonucleotide primers shown in Table S2. Each sample was genotyped in duplicate 10-μL reactions containing 1 μL DNA extract, 0.14 μL KASP™ Assay Mix and 5 μL 2× KASP™ Master Mix. Each assay run also included no-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 10 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 of 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

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

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(WT) SNP genotype with deltamethrin susceptibility, and the mutant (Mu) SNP genotype with deltamethrin resistance.

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.[29] 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 heterozygote 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.

2.8 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 Core Team (Vienna, Austria, 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 ($LsNa_v1.3$), 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 (Michel Raymond and Francois Rousset, Laboratiore de Genetique et Environment, Montpellier, France). The significance level was set at $P < 0.05$.

3 RESULTS

3.1 Susceptibility of *L. salmonis* strains to etofenprox

Lepeophtheirus salmonis strains studied in this report have been characterised previously. Strain IoA-00 is drug-susceptible, whereas strain IoA-02 is 143-fold resistant to deltamethrin[21] and further shows hyposensitivity to emamectin benzoate and azamethiphos.[24,30] Strain IoA-02 shows a high allele frequency of the putative *kdr* mutation $LsNa_v1.3$ A3041G, whereas all tested individuals of the IoA-00 strain show the wild-type allele at this locus (Table 1). IoA-00 and IoA-02 showed similar susceptibility to the non-ester pyrethroid etofenprox (Table 2).

3.2 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.[21] In a family derived from an IoA-00 female and an IoA-02 male, 20% of F2 parasites were resistant (Fig. S1).[21] In the current study, an available archived F2 specimen of the experiment, as well as parental strain *L. salmonis*, were genotyped at the SNP loci $LsNa_v1.3$ A3041G and mtDNA A14013G (CytB) and A9030G (COX1) (Table 1). Confirming earlier reports,[21] 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 $LsNa_v1.3$ SNP A3041G locus, all conceivable genotypes were observed in F2 animals. Genotype and allele

Table 1. Genetic association of nuclear and mitochondrial single nucleotide polymorphisms (SNPs) with deltamethrin resistance in *Lepeophtheirus salmonis* laboratory strains and their F2 progenies

		Laboratory strains		F2 generation derived from cross	
		IoA-00	IoA-02	IoA-02 male × IoA-00 female	
		Susceptible[a]	Resistant[a]	Susceptible[b]	Resistant[b]
n		14	16	55	13
$LsNa_v1.3$ 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

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. S1). 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 $LsNa_v1.3$) and mitochondrial SNPs A14013G (cytochrome *B*; CytB) and A9030G (cytochrome *c* oxidase subunit I; COX1).

[a] 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.

[b] 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).

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Table 2. Susceptibility of laboratory *Lepeophtheirus salmonis* strains to etofenprox

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[a]	**1.31**	**0.81**

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 followed by different letters for each pesticide are significantly different ($P < 0.05$).
[a] Resistance ratio of etofenprox: EC_{50} IoA-02/EC_{50} IoA-00.

Table 3. Susceptibility of *Lepeophtheirus salmonis* from different Scottish aquaculture production sites to deltamethrin

Scottish county	Year	EC_{50} (μg L^{-1})[a] and 95% confidence limits
Sutherland 17	2017	1.60 (0.91–2.29)
Argyll 18	2018	>2.0[b]
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)

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.
[a] Raw data used to determine median effective concentrations (EC_{50}) are provided in Table S1.
[b] Single-dose bioassay: 94.4% (N = 54) remained unaffected after exposure to 2 μg L^{-1} deltamethrin.

frequencies of $LsNa_v1.3$ SNP A3041G did not differ between deltamethrin-resistant and deltamethrin-susceptible F2 individuals ($P > 0.05$).

3.3 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 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 (Tables 4 and 5).

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

Genotype and allele frequencies of the $LsNa_v1.3$ SNP A3041G did not differ ($P > 0.05$) between deltamethrin-resistant and -susceptible salmon lice from field populations (Table 4). Na_v target-site resistance has been shown to be inherited either as a recessive or incomplete recessive trait,[31–35] 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. By 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 5). Genotypic classification of deltamethrin resistance based on mtDNA markers A14013G and A9030G showed a 79% compliance with phenotypic classification based on bioassays.

Table 4. Genetic association of single nucleotide polymorphisms A3041G in voltage-gated sodium channel homologue $LsNa_v1.3$ with deltamethrin (DM) resistance in *Lepeophtheirus salmonis*

Origin of L. salmon*is*	DM resistance[a]	*n*	Genotype frequencies A/A	A/G	G/G	G Allele	*P*-value[b] Genotypic/allelic differentiation	CS (%) for recessive inheritance of resistance trait[c]	CS (%) for incomplete recessive inheritance of resistance trait[c]
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[b]	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			

Salmon lice from Scottish aquaculture sites were classified as deltamethrin-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 deltamethrin sensitivity.
[a] 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).
[b] 21 of 37 deltamethrin-resistant individuals were subjected to allele specific PCR genotyping at SNP A3041G.
[c] 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.

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Table 5. Genetic association of mitochondrial single nucleotide polymorphisms A14013G (cytochrome *B*; CytB) and A9030G (cytochrome *c* oxidase subunit I; COX1) with deltamethrin (DM) resistance in *Lepeophtheirus salmonis*

			Allele frequencies			
Origin of *L. salmonis*	DM resistance[a]	*n*	A	G	*P*-value[b] Allelic differentiation	CS (%) of 14013G or 3090G
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[b]	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		

Salmon lice from Scottish aquaculture sites were classified as deltamethrin-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 DM sensitivity.
[a] 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).
[b] 21 of 37 deltamethrin-resistant individuals were subjected to allele specific PCR genotyping at SNPs A14013G and A9030G.

4 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* Vssc1) has been suggested as a potential genetic determinant of pyrethroid resistance in this species.[18] 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.[21,22] The current 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).[36] *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.[37–40] To date, > 50 *kdr* mutations/combinations of *kdr* mutations have been identified, some of which evolved independently in different arthropod species.[41] 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[42] 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[43] and harbours the first isolated *kdr* mutation L1041F (numbering according to *M. domestica* Vssc1).[44] 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.[42,43] Electrophysiological characterisation of mutant *Drosophila melanogaster* (Meigen) Na_v expressed in *Xenopus* oocytes revealed that the mutation I936V reduces the channel's sensitivity to deltamethrin.[20] 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 with parasites expressing the wild-type channel.

To distinguish potential effects of I936V $LsNa_v1.3$ from those of the mitochondrial haplotype associated with deltamethrin resistance,[21,22] 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.[21] By contrast, in families derived from crosses of the inverse orientation (IoA-00 female × 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 $LsNa_v1.3$ allele or genotype frequencies between deltamethrin-resistant and drug-susceptible F2 parasites. This finding suggests that the $LsNa_v1.3$ locus was not a genetic determinant of deltamethrin resistance in the cross. The reason for the lack of association of the I936V $LsNa_v1.3$ mutation with deltamethrin susceptibility in the cross is unknown. The residue Ile936 lies in the highly conserved S5 helix of DII, which forms part of the first proposed pyrethroid binding site of Na_v.[42] Three conserved residues of arthropods Na_v, Cys933, Ile936 and Phe1530, show a divergent substitutions at the homologous positions of the vertebrate Na_v (Ala, Val and Ile, respectively), and have been suggested to contribute to the lower affinity of the vertebrate channel to pyrethroids.[42] 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.[20] Accordingly, it appears likely that I936V may also affect deltamethrin in

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the context of LsNa$_v$1.3. However, the cellular localisation and functional role of LsNa$_v$1.3 is unknown, and the channel may have no or only secondary relevance as a molecular target site for acute toxic effects of deltamethrin in adult and pre-adult *L. salmonis*. Deltamethrin toxicity in these parasite states could potentially be mainly mediated through other molecular targets, such as further Na$_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.[45] Ligand-docking studies on a *M. domestica* Na$_v$ model revealed similar binding positions of fenvalerate, the base molecule for etofenprox, and deltamethrin.[20,42] 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.[46–49] In the current study, the deltamethrin-resistant *L. salmonis* strain IoA-02 did not differ in susceptibility to etofenprox when compared with 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 (1.6–8.0 μg L^{-1}) were significantly higher than values reported previously for the deltamethrin-susceptible IoA-00 strain (0.28 μg L^{-1}, 95% confidence limits 0.23–0.36 μg L^{-1}),[21] suggesting that deltamethrin resistance is widespread in *L. salmonis* populations of the Scottish west coasts, confirming the results of earlier studies.[11,21] 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$_v$1.3 I936V mutation and two mitochondrial SNPs allowing detection of the pyrethroid resistance-associated mitochondrial haplotype. Deltamethrin-resistant and -susceptible *L. salmonis* obtained from field sites did not differ in allele and genotype frequencies for the LsNa$_v$1.3 I936V locus, which is in line with the above results obtained from genotyping of resistant and susceptible F2 parasites, but contrasts previously published findings.[18]

In contrast to LsNa$_v$1.3 I936V, both mitochondrial markers A14013G (CytB) and A9030G (COX1) significantly differentiated deltamethrin-resistant and -susceptible parasites for all aquaculture production sites, except for one population in which very few susceptible parasites had been obtained, lowering testing power. These results support the hypothesis that deltamethrin resistance in Scottish field populations of *L. salmonis* involved mitochondrial genetic determinants and can be reliably monitored by mitochondrial SNP markers proposed in earlier studies.[21] The mtDNA SNPs A14013G and A9030G used in this study are markers of a previously defined deltamethrin resistance associated mtDNA haplotype involving further 26 SNPs.[21] It has been suggested that the resistance-associated mitochondrial haplotype first emerged around 2009, when it was detectable in parasites from aquaculture sites in Ireland, the Shetland isles and Norway.[11] However, moderate levels of deltamethrin resistance had already been reported in the early 2000s, with EC$_{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.[50]

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*,[21,23] 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,[51] the formation of secondary structures of proteins,[52] protein folding and substrate/protein interaction.[53] 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$_v$[15,16] and enhanced enzymatic detoxification.[17] 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.[18] The current study provides an in-depth investigation of LsNa$_v$1.3 I936V, a SNPs identified in the previous study, but results argue against relevance of this mutation for deltamethrin resistance in the studied parasites. In terrestrial arthropods, pyrethroid resistance based on metabolic detoxification usually involves enhanced expression of biotransformation enzymes, such as CYPs, esterases or GSTs.[54] For example, pyrethroid resistance in *Anopheles funestus* (Giles) did not involve Na$_v$ mutations, but was linked to overexpression of CYPs and GSTs.[55–57] Similarly, metabolic resistance has been implicated in pyrethroid resistance in isolates of *Aedes albopictus* (Skuse),[58] *Anopheles arabiensis* (Patton)[59] and *M. domestica*.[60] Several studies have investigated the transcriptional responses of caligid sea lice to pyrethroid exposure, demonstrating effects on transcript expression of CYPs,[30,61] serine proteases[61,62] and antioxidant enzymes.[61,63] 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.[30] Insect populations in which pyrethroid resistance is based on CYP overexpression often show cross-resistance to etofenprox,[64,65] whose chemical structure impedes its metabolic detoxification by esterases and GSTs.[66,67] 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*.

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5 CONCLUSION

In this 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/pre-adult 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*.

ACKNOWLEDGEMENTS

This work was supported by a PhD studentship awarded to Claudia Tschesche by PHARMAQ/Zoetis. This study was further supported by United Kingdom Biotechnology and Biological Sciences Research Council grant BB/L022923/1 awarded to Armin Sturm. The authors further gratefully acknowledge funding received from the MASTS pooling initiative (The Marine Alliance for Science and Technology for Scotland). MASTS is funded by the Scottish Funding Council (grant reference HR09011) and contributing institutions. The authors are further thankful to the Scottish Salmon Producer's Organisation for financial support. The authors are thankful to Iain McEwen from Fish Vet Group UK for valuable help in performing salmon lice bioassays with deltamethrin and the provision of sea louse samples. The funders had no role in study design, data analysis or interpretation of results.

SUPPORTING INFORMATION

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

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