Key Role of Mitochondrial Mutation Leu107Ser (COX1) in Deltamethrin Resistance in Salmon Lice (*Lepeophtheirus Salmonis*)

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Abstract

Deltamethrin (DTM) is used to treat Atlantic salmon (*Salmon salar*) against salmon lice (*Lepeophtheirus salmonis*) infestations. However, development of DTM resistance has been reported from North Atlantic *L. salmonis* populations, in which resistance is associated with mitochondrial (mtDNA) mutations. This study investigated the relationship between DTM resistance and mtDNA single nucleotide polymorphisms (SNPs). A total of 188 *L. salmonis* collected from Scottish aquaculture sites were assessed using DTM bioassays and genotyped at 18 SNP loci. Genotyping further included archived parasites of known DTM susceptibility status. The results identified eleven mtDNA haplotypes, three of which were associated with DTM resistance. Phylogenetic analyses of haplotypes suggested multiple origins of DTM resistance. *L. salmonis* laboratory strains IoA-00 and IoA-10 showed similarly high levels (~100-fold) of DTM resistance in bioassays. Both strains differed strongly in mtDNA haplotype, but shared the missense mutation Leu107Ser in the mitochondrial gene cytochrome c oxidase subunit 1 (COX1), which was detected in all further DTM resistant *L. salmonis* isolates assessed. In crossing experiments with a DTM-susceptible strains, maternal inheritance of DTM 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 DTM resistance in *L. salmonis.*

Introduction

Caligid sea lice (Copepoda: Crustacea) are ectoparasite of marine fish, which feed on the mucus, skin and blood of their hosts [1] . 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 [2,3] . Sea lice infections are a major health management problem in the commercial mariculture of Atlantic salmon (*Salmo salar* L.), with the salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837) 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 [4] , comprising mainly treatment costs and to a lesser extent losses 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 [5,6]. Non-medicinal sea lice control approaches include alternative cage designs reducing infection pressure [7], different systems of salmon delousing using physical means such as the application of water jets or brief immersion in warm water [8], and the co-culture of Atlantic salmon with different species of cleaner fish that remove sea lice from infected salmon [9]. 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 (DTM), which are supplied as bath treatments [5].

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[6,10,11]. The pyrethroid DTM 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[12], higher levels of resistance have been described more recently[13]. The toxicity of pyrethroids is based on their blocking of arthropod voltagegated sodium channels (Na_v), which have essential roles in neurotransmission[14]. Pyrethroid resistance in insects can be based on missense mutations of Na_v that decrease the channel's affinity to the pesticides[15,16], 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[17].

In *L. salmonis*, the molecular mechanism of pyrethroid resistance remains to be resolved. DTM resistance in *L. salmonis* shows a predominantly maternal mode of inheritance, and is associated with mitochondrial mutations[13,18]. These findings suggest a novel, still unresolved resistance mechanism and imply still unidentified mitochondrial targets for DTM toxicity in susceptible lice. In support of this hypothesis, DTM has been shown to disrupt mitochondrial ATP production[13] and induce apoptosis in skeletal muscle, which have a high content of mitochondria[18]. Results from crossing experiments suggested additional minor roles of unidentified nuclear genetic factors in DTM susceptibility[13]. In *L. salmonis*, three genes encoding Na_v channels have been identified[19], one of which harbours a single nucleotide polymorphism (SNP) that is homologous to a mutation of insect Na_v decreasing pyrethroid affinity[20]. However, in *L. salmonis* field populations the Na_v SNP showed no association with DTM resistance[21]. 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 DTM resistant parasites[21,22].

In pest or parasite populations, mutations causing pesticide resistance can form *de novo* or be introduced through migration[23]. 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[24,25]. 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[26], 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[27].

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[25]. In contrast, the analysis of SNPs closely linked to a mutation in the *L. salmonis ace1a* gene, previously shown to confer azamethiphos resistance[28], revealed multiple and divergent haplotypes associated with resistance[29]. 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 DTM 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 DTM susceptible samples[13]. While these findings suggest the clonal expansion of DTM-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 DTM resistance. *L. salmonis* were obtained from a range of Scottish farm sites and subjected to bioassays to determine their DTM susceptibility status. Parasites were then genotyped at selected mitochondrial SNP loci to identify mitochondrial haplotypes and assess haplotype association with DTM resistance. Mitochondrial haplotypes were compared to infer whether DTM 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 DTM resistant and susceptible *L. salmonis* strains to derive hypotheses about the potential involvement of mitochondrial SNPs in the resistance mechanisms. The DTM susceptibility of the newly established strain was characterised using drug bioassays and effects of sublethal DTM concentrations on total ATP levels were assessed. The strain was further subjected to crossing experiments to assess the mode of inheritance of DTM resistance.

Materials And Methods

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 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 license and at low parasite densities unlikely to compromise fish welfare.

Lepeophtheirus salmonis **strains and husbandry**

L. salmonis strains were maintained under standardized conditions at the Marine Environmental Research Laboratory of the University of Stirling (Machrihanish, UK), as described in detail elsewhere[30].

Strain IoA-00 was taken into culture in 2003 and is susceptible to all current salmon delousing agents, while strain IoA-02 was established in 2011 and is resistant to emamectin benzoate, DTM, and azamethiphos[13,22,31]. Strain IoA-10 was established during this study. To establish this strain, gravid female *L. salmonis* were obtained from an aquaculture production site located on the West coast of Scotland, put into flasks containing cold oxygen-saturated seawater and shipped to the laboratory. Paired egg strings were removed from each parasite and incubated in separate containers as described before to allow hatching and progression to the copepodid stage. The corresponding females were preserved in absolute ethanol and subjected to genotyping assays to establish mitochondrial haplotypes. Based on the results, larvae derived from one dam showing a mitochondrial haplotype of interest were selected and used to infect naïve host fish in order to establish strain IoA-10.

This study further included archived *L. salmonis* from nine aquaculture production sites, as well as from wild host fish caught in a salmon river located on the East coast of Scotland, which were stored in absolute ethanol at -20°C pending molecular analyses.

L. salmonis **bioassays**

Bioassays were performed to assess the susceptibility of both preadult-II/adult salmon lice and copepodid larvae lice to DTM (Pestanal® analytical standard grade, Sigma-Aldrich, 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 temperaturecontrolled chamber set to 12°C.

Bioassays with preadult/adult salmon lice performed within this study had either a standard or a singledose design[12,32], and have been described in detail elsewhere[21]. 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 DTM concentrations in the range of 0.125 and 32 μg/L and a solvent control (Supplementary Table S1) in duplicates. Singledose bioassays were conducted in an analogous fashion, except that DTM was provided at one diagnostic concentration (2 μg/L). Preadult/adult salmon lice were exposed to DTM 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[31].

To generate *L. salmonis* copepodid larvae for use in bioassay, paired egg strings of gravid females were incubated in aerated filtered seawater in a temperaturecontrolled chamber set to 12°C, while keeping samples separate. 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 DTM to the embryo dishes containing 2 mL seawater and parasites. Copepodids were exposed to nine concentrations of DTM (0.10, 0.22, 0.46, 1.00, 2.15, 4.64, 10.00, 21.54, and 46.42 μg/L) and one solvent control, in duplicates. DTM 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 singledose bioassays involving exposure to 2 µg/L DTM parasites were classified as DTM 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.

Genotyping of mitochondrial single nucleotide polymorphism (SNP) alleles

PCR based genotyping assays employing universal fluorescence energy transfer (FRET) probes (KASP® 4.0, LGC Genomics, UK) were designed to detect 18 mtDNA SNPs, including the four nonsynonymous SNPs G8134A, T5889C, T8600C, and G3338A, that have been associated with DTM resistance in *L. salmonis* in a previous study[13]. Genomic DNA was extracted from individual *L. salmonis* specimens using a high throughput protocol[33], with details having been reported elsewhere[21]. Each SNP assay involved one common primer and two allele specific primers (Supplementary Table S2), with the method being described in detail elsewhere[21].

Mitochondrial haplotype network

A haplotype network was interfered from mitochondrial haplotypes of *L. salmonis*. The network was constructed using the medium-joining method[34] implemented in the software PopArt v1.7[35]. Haplotypes were defined based on the combined occurrence of 18 SNPs that have been associated with DTM resistance in *L. salmonis* in a previous study[13].

Mitochondrial genome (mtDNA) amplification and sequencing

The mitochondrial genome was amplified and sequenced from two salmon lice from the laboratorymaintained 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 (Supplementary Table S3). PCR reactions were performed using 1 µl of 50 ng/µL 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 Supplementary Supplementary Table S4. All PCR products were purified (NucleoSpin ™ Gel and PCR Cleanup Kit, Macherey-Nagel, Düren, Germany) and sequenced (Supplementary Table S5) by

Eurofins Genomics (Ebersberg, Germany). Prior to sequencing, PCR product 6 (Supplementary Table S3) had to be subcloned using a pGEM-T Easy Vector system and chemocompetent *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.

Identification of DTM 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)[13], were aligned to the Scottish *L. salmonis* mitochondrial reference genome (GenBank: LT630766.1) using the R/Bioconductor package Rbowtie2 v 4.0.3[36]. Sequence variations were identified using the *HaplotypeCaller* function in GATK v3.5[37]. IoA-10 specific SNPs, as well as SNPs common to all DTM resistant individuals and lacking in all susceptible parasites were then identified.

L. 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 preadultII 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 preadultII 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; 99%; Sigma-Aldrich, U.K.) 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/preadultII 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[38]. 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 DTM susceptibility phenotype.

Effects of DTM 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 DTM and fenpyroximate (Pestanal® analytical standard grade, Sigma-Aldrich, UK). The acaricide fenpyroximate served as a positive control, as it is known to act by interference with the mitochondrial complex I[39].

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. DTM and fenpyroximate were solubilized in acetone (0.05% v/v in all tests). Parasites were exposed to a solvent control and sublethal concentrations of the tested compounds (DTM: 2 μg/L; fenpyroximate: 100 μg/L) in quadruplicate test dishes. Adult males were exposed to DTM 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, UK; Thistle Scientific Ltd, 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 luciferinluciferase bioluminescence assay kit (A-22066, Molecular Probes, Thermo Fisher Scientific, UK), with details having been reported elsewhere[13].

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

Amino acid sequences of ND1, ND5, COX1, and COX3 from *L. salmonis* and other crustaceans (GenBank 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, *Tetraclita japonica* NC_008974.1, *Homarus americanus* NC_015607.1, *Vargula 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 v2.1[40].

Data analyses and statistical tests

The dose-response relationship for DTM tested in *L. salmonis* bioassays was assessed by probit analysis using R package *drc* (v3.0-1), assuming a lognormal 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 packages car v3.0-11, rcompanion v2.4-1 and PMCMR v4.3. 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 experimentwise type I error was controlled by sequential Bonferroni correction[41]. 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.

Results

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

L. salmonis collected at Scottish aquaculture sites were subjected to bioassays involving exposure to 2 µg/L DTM, with surviving animals failing to show behavioural responses being classified as DTM resistant and animals showing mortality or behavioural responses classified as DTM susceptible. Resistant and susceptible animals from field sites, as well as parasites from previously characterized laboratory strains (IoA-00, DTM susceptible; IoA-02, DTM resistant), were genotyped at four nonsynonymous mtDNA SNP loci, which have previously been shown to be associated with DTM susceptibility[13]. The SNPs used comprised mtDNA mutations G8134A, T5889C, T8600C, and G3338A, which are located in mitochondrial genes NADH dehydrogenase subunit (ND1), NADH dehydrogenase subunit (ND5), cytochrome c oxidase subunit (COX1), and cytochrome c oxidase subunit 3 (COX3), respectively. Based on the combined occurrence of these SNPs, four haplotypes were defined (Table 1). As reported previously, lice of the DTM susceptible strain IoA-00 possess haplotype 1, defined by the wild type alleles T8600, G8134, T5889, and G3338, whereas parasites from the DTM resistant strain IoA-02 show haplotype 2, comprising mutant alleles 3338A, 5889C, 8134A, and 8600C[13]. Genotyping of salmon lice from field populations revealed, in addition to haplotypes 1 and 2, two new haplotypes 3 and 4. Most animals with haplotype 2, 3 and 4 were classified as DTM resistant (93%, 87% and 100%, respectively). In contrast, 57% of individuals with haplotype 1 were rated DTM susceptible, while 43% were classified resistant (Table 2).

Table 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[13].

Position[a]	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

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

Table 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 1 for details).

Origin of *L. salmonis*	Deltamethrin resistance[a]	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[b]	-	16	3	2
Farm site 4	Susceptible	10	6	3	1	-
(Sutherland, 2019)	Resistant	20	1	15	4	-
Total (Farm sites)		218	N = 14	N = 157	N = 31	N = 16
	Susceptible	23	57.1%	7%	12.9%	0%
	Resistant	195	42.9%	93%	87.1%	100%

[a] Susceptibility to deltamethrin was determined in single-dose bioassays, involving exposure (30 min) to 2 µg/L deltamethrin, followed by recovery in seawater (24 h) and subsequent rating as susceptible (affected) or resistant (unaffected). [b] 21 out of a total of 37 deltamethrin resistant individuals observed were successfully genotyped.

Relationship of mtDNA haplotypes to DTM resistance of copepodids

The relationship between mtDNA haplotypes and DTM 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 DTM EC_{50} in haplotype 1 copepodids (laboratory strain IoA-00) was determined as 0.14 µg/L. For copepodid larvae possessing haplotype 2, estimates of the DTM EC_{50} value were 4.81 µg/L (laboratory strain IoA-02) and 3.98 µg/L (field derived larvae), while copepodids having haplotype 3 showed an EC_{50} of 6.90 µg/L, field derived larvae (Table 3).

Table 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*	Num bioassay	Haplotype[a]	EC_{50} (95% CI)[b] [µg/L]
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)

[a] For definition of haplotypes, see Table 1. [b] Raw data used to derive EC_{50} values are provided in Supplementary Table S10.

Further characterization of mitochondrial haplotypes

In order 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 DTM resistance[13] (Supplementary Table S6). Genotyping further included *L. salmonis* collected from wild hosts in 2010, as well as conserved specimens of the DTM resistant strain NA01-O[13]. 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 8 new haplotypes named 1A to 1H (Supplementary Table S6). Resistanceassociated haplotype 4 was found to be present in specimens removed from wild fish in 2010 and both resistanceassociated 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 DTM resistance in bioassays, was closely related to susceptible haplotypes (1A, 1D-F, and 1H), but phylogenetically distant to the DTM resistanceassociated haplotypes 2 and 4 (Fig. 1). Further experiments focused on characterising the DTM 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 (Supplementary Table S6).

Establishment and characterization 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 (Supplementary Table S7). The mtDNA genome of IoA-10 strain lice was compared to that of previously analysed DTM resistant, IoA-02, IoA-03, NA01-O, NA01-P, and DTM susceptible *L. salmonis* strains, IoA-00, IoA-01[13], in order to identify SNPs associated with DTM resistance. The nonsynonymous 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 4).

Table 4
Mitochondrial sequence variations specific for deltamethrin resistant L. salmonis **isolates**. The mitochondrial genome (mtDNA) was compared between deltamethrin resistant (IoA-02, IoA-03, IoA-10, NA01-O, NA01-P) and susceptible (IoA-00, IoA-01) *L. salmonis* strains to establish mutations associated with resistance. The mtDNA sequence of strain IoA-10 was established by PCR followed by Sanger sequencing within the present study, while that of the other strains has been reported previously[13].

Position[a]	Type	Location	Description	Reference	Alternative
812	Polymorphism	D-loop		T	C
875	Polymorphism	D-loop		T	C
940	Deletion	D-loop		AG	A
960	Polymorphism	D-loop		T	C
963	Polymorphism	D-loop		G	A
972	Polymorphism	D-loop		A	G
8600	Non-synonymous mutation	COX1	TTG/Leu → TCG/Ser	T	C
10178	Polymorphism	l-rRNA		A	G
15377	Polymorphism	D-loop		G	A

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

To assess the mode by which DTM 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 (filial generation 1) lice (Supplementary Table S9). The DTM susceptibility of parental and F1 populations was determined in bioassays (Supplementary Table S11). Strains IoA-02 and IoA-10, as well as F1 offspring derived from IoA-10 dams and IoA-00 sires, were highly DTM resistant (EC$_{50}$ values > 24.0 µg/L; Fig. 2). In contrast, F1 animals derived from IoA-00 dams and IoA-10 sires (EC$_{50}$ 0.55 µg/L; Fig. 2) were only marginally but significantly (P < 0.0001) less susceptible to DTM than IoA-00 lice (EC$_{50}$ 0.25 µg/L; Fig. 2).

To further characterise DTM toxicity in IoA-10 lice, the effects of DTM exposure on wholebody ATP levels in strain IoA-10 were investigated. Experiments further included fenpyroximate (100 µg/L), an acaricide known to block oxidative phosphorylation. As expected, fenpyroximate caused toxicity, and decreased whole-body ATP levels in all strains assessed (Supplementary Table S10 & Fig. 3). Exposure to 2 µg/L DTM 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.

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 for which mtDNA sequences were available were aligned to reveal conserved residues within these mitochondrial polypeptides. Among four nonsynonymous mtDNA SNP loci that have previously been reported from a DTM resistant strain[13], 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 (Supplementary Fig. S1).

Discussion

Previous studies have shown that DTM resistance in *L. salmonis* is transmitted predominantly by maternal inheritance and associated with mtDNA SNPs[13,42]. While an earlier study suggested that highly DTM resistant *L. salmonis* share virtually identical mtDNA sequences[13], 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 DTM resistance associated haplotypes, showed a high level of DTM resistance comparable to that of the previously characterised strain IoA02 and, similar to IoA02, passed on DTM resistance to the next generation through maternal inheritance. The phylogenetic analysis of mtDNA haplotypes provided evidence for multiple origins of mtDNAassociated DTM resistance in *L. salmonis*. Comparison of mtDNA sequences between DTM resistant and susceptible salmon louse strains suggested the association of DTM resistance with SNP T8600C, corresponding to Leu107Ser in COX1.

L. salmonis obtained at aquaculture sites were subjected to bioassays to determine their DTM susceptibility status. Parasites were then genotyped at previously described mtDNA SNP loci[13] to identify mitochondrial haplotypes and assess haplotype association with DTM resistance. 93%, 87%, and 100% of lice possessing haplotypes 2, 3, and 4, respectively, were classified as DTM 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[10,43]. 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 DTM resistance phenotype was less distinct for lice containing haplotype 1. While most lice with this haplotype were classified as DTM 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 DTM resistance phenotype, which is in line with findings by Carmona-Antoñanzas et al.[13] who attributed the presence of about 20% resistant F2 parasites in a family descending from a DTM 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 [44] and were hatched and reared under standardized 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 DTM resistance. Moreover, the study showed that DTM resistance is already present in the larval stage, which would be expected for resistance conferred by mtDNA mutations[13].

Genotyping of field isolates collected in 2018 and 2019 revealed three DTM 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 DTM resistant strain NA01-O, which was established in 2013. These findings contrast with results from Carmona-Antoñanzas et al.[13], who reported mtDNA sequences consistent with haplotype 2 for all DTM 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[13] could be attributed to the limited number of sequenced individuals.

Phylogenetic analyses of mtDNA haplotypes showed that the three DTM 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 DTM resistant isolates indicate that mtDNA-associated DTM resistance in *L. salmonis* originated from at least two independent origins. Thus, resistanceassociated 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 DTM 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 DTM resistant (EC_{50} values >24.0 µg/L; P = 0.845) and reciprocal crosses of strains IoA-10 (present study) and IoA-02[13] with the drugsusceptible IoA00 lice revealed that both strains transmit their resistance to the next generation through maternal inheritance. Moreover, DTM exposure caused behavioural toxicity and wholebody ATP depletion in DTM 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 DTM exposure on behavioural toxicity and ATP levels between IoA-00 and IoA-02 lice[13]. The maternal inheritance of DTM 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[13]. Depletion of ATP levels in DTM susceptible lice may be related to the toxic effect of DTM 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 DTM resistance are transmitted together with irrelevant hitchhiking SNPs[44]. Thus, SNPs that are truly linked to DTM resistance are expected to be present in all resistanceassociated haplotypes but lacking in all susceptibilityassociated haplotypes. The nonsynonymous 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 DTM 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[45]. 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[46]. 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.[18]. Mitochondrial rRNAs are assembled with ribosomal proteins encoded by nuclear genes to form mitochondrial ribosomes, which are responsible for translating mitochondrial proteins[47]. Thus, mutation within the mitochondrial rRNA may lead to ribosome dysfunction and may result in respiratory chain defects [48]. 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 DTM resistance and by inference the mechanism of DTM toxicity in *L. salmonis*. While it is generally accepted that pyrethroids target Na_v in terrestrial arthopods[14], 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[49]. For example, pyrethroids have been shown to affect mitochondrial membrane structures and dynamics, which can impair oxidative phosphorylation[50,51]. Mitochondrial oxidative phosphorylation can also be impaired by intracellular Ca2+ accumulation[52,53], which can result from interactions of pyrethroids with Nav and consequent Ca2+ influx[54] and direct effects of pyrethroids on voltage-gated Ca2+ channels[55].

Pyrethroid induced disruption of mitochondrial membrane integrity and inhibition of respiratory complexes, as well as intracellular Ca2+ accumulation can cause the generation of reactive oxygen species (ROS) in mitochondria[56–58]. ROS can trigger a cascade of reactions that induces lipid peroxidation and damage of macromolecules[59–61]. Pyrethroids have been shown to induce intrinsic mitochondrial apoptosis[62,63], which involves mitochondrial outer membrane permeabilization, release of cytochrome C into the cytosol, activation of caspases, and ultimately DNA fragmentation[64]. 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[65–68]. Interestingly, DTM 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 DTM resistant strain[18].

Taken together, in salmon lice, DTM 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 DTM toxicity in salmon lice would be that DTM 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[56]. Both, low levels of ATP and oxidative stress can in turn induce intrinsic mitochondrial apoptosis[65,66,68], which has been described by Bakke et al.[18]. Resistance may be conferred by mitochondrial SNP(s) that changes the amino-acid sequences of the complex and impair binding of DTM. For reasons explained above, T8600C leading to Leu107Ser in COX1 is the most probable mutation for conferring DTM resistance in salmon lice. Alternatively, DTM might impair the mitochondrial ATP production in susceptible lice by causing disruptions of the mitochondrial membrane or by secondary effects arising from DTM toxicity. In these scenarios, mtDNA mutation(s) may have functional effects on the efficiency of electron transfer or proton translocation, counteracting ATP deficits.

Conclusion

DTM 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 DTM 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 DTM resistant and pass on their resistance to the next generation through maternal inheritance. The results suggest the association of DTM resistance with SNP T8600C (Leu107Ser in COX1).

Declarations

Acknowledgments

Author contributions

All authors conceived the experiment, C.T. conducted the experiment, C.T., A.S and M.B. analysed the data. C.T., A.S., M.B. and J.E.B. reviewed and discussed the results. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Data availability

The sequencing data were submitted to the EBI ENA database under the Project number PRJEB47839.

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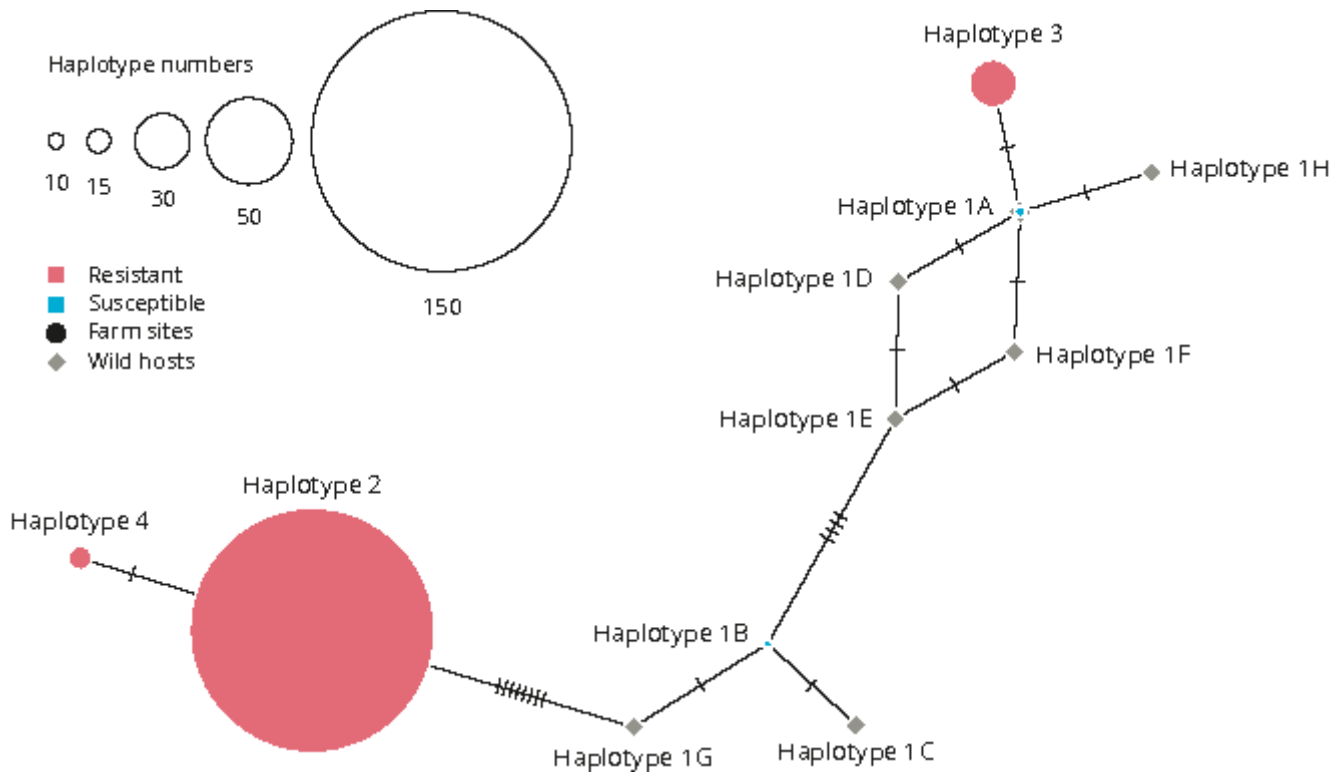
Figures

Figure 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 study13 (Supplementary Tables S6 and S7). 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.
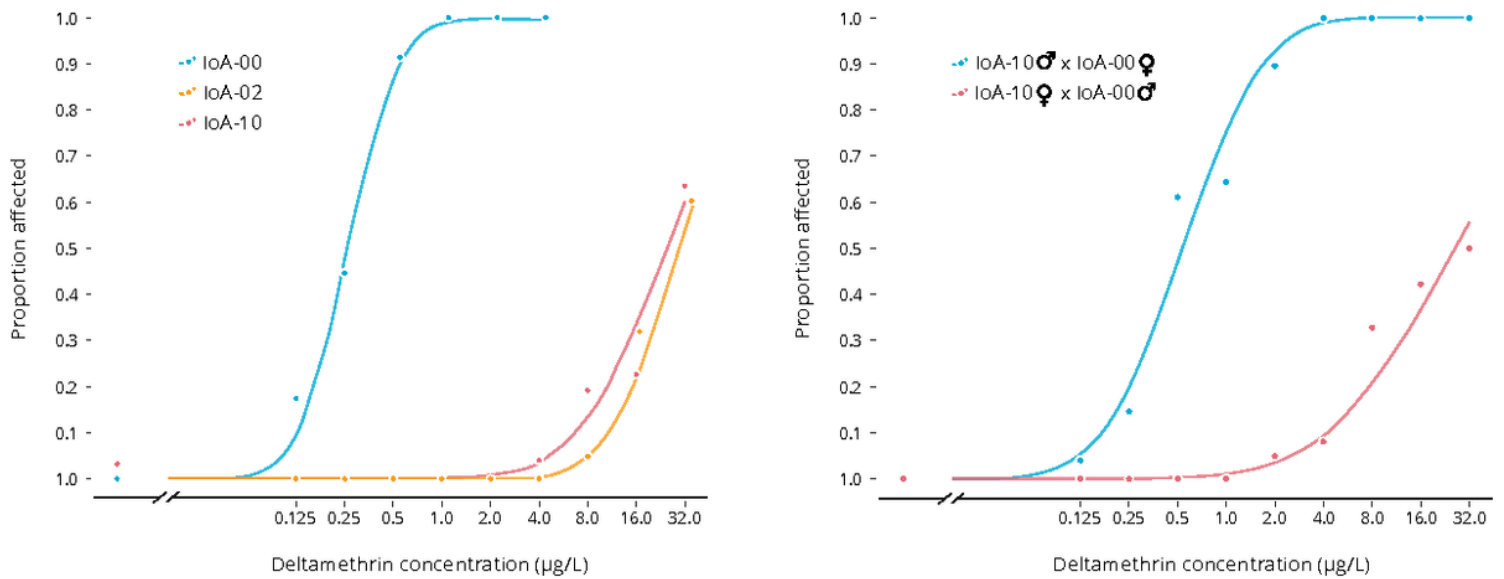
Figure 2

Standard deltamethrin bioassay with L. salmonis first filial (F1) progenies derived from parental (P0) crosses of different gender-strain orientations. Median effective concentrations EC50 [µg/L] 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 Supplementary Table S11.
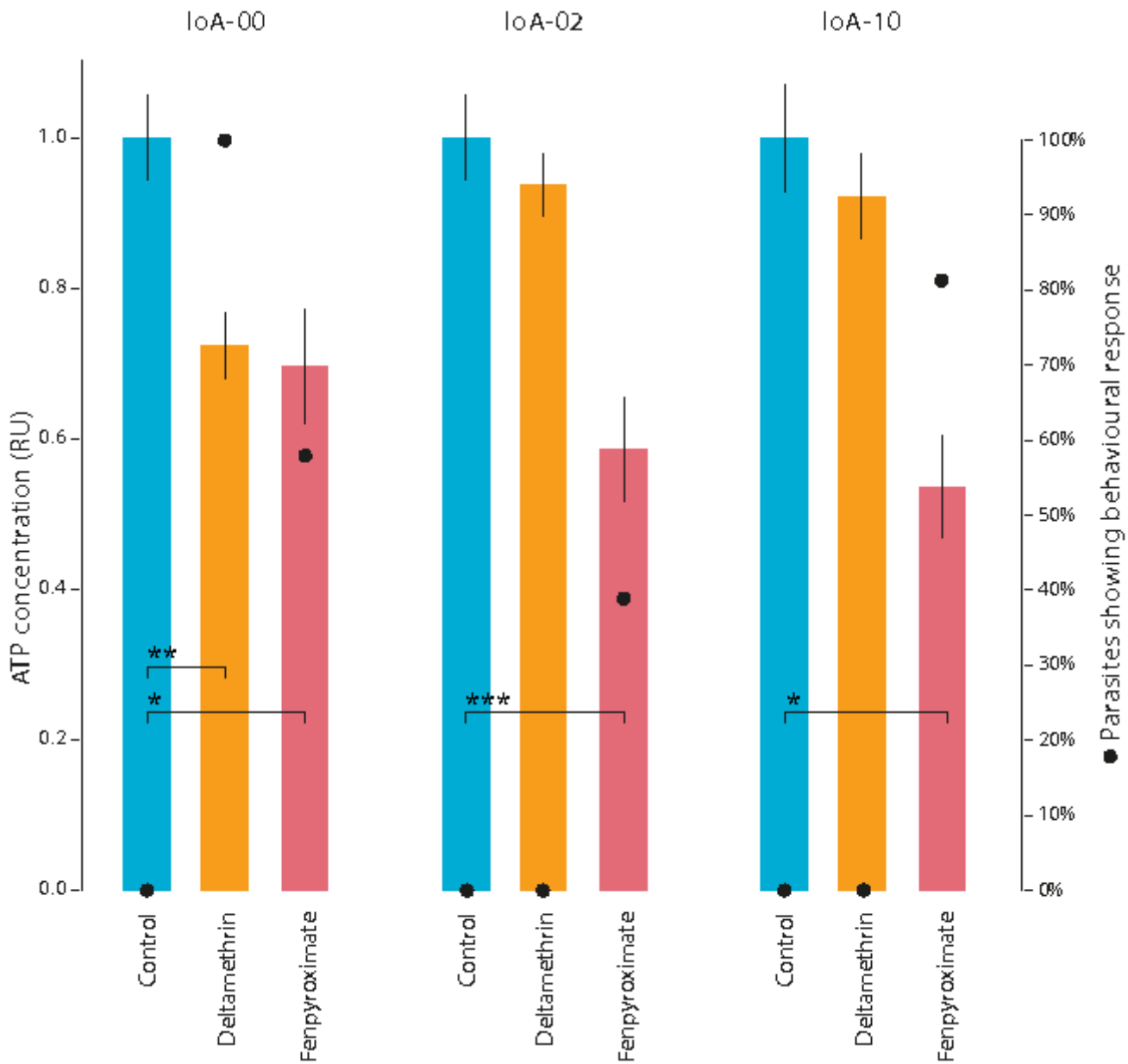
Figure 3

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), fenpyroximate (100 µg/L), or a solvent acetone control (0.05% v/v, control) for 300 min before behavioural effects were recorded (secondary y-axis; orange 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).

Supplementary Files

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